THE ROLE OF LIPOCALIN 2 IN WHITE ADIPOSE TISSUE BEIGING AND METABOLIC HEALTHSPAN

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Dedication

This dissertation is dedicated to my husband, Christopher Scott.

Abstract

Adipose tissue dysfunction in response to obesity or aging is a major underlying factor in the development of metabolic diseases. Therefore, identifying mechanisms that promote adipose tissue health in the face of metabolic challenges is essential for preventing further deterioration of whole-body metabolic homeostasis. Lipocalin 2 (Lcn2) is an adipose tissue-derived secreted protein that has previously been shown to play a role in diet-induced obesity, metabolic disease, and brown adipose tissue (BAT) thermogenesis. This thesis project addresses two main questions: 1) whether Lcn2 regulates thermogenic activation, or beiging, of inguinal white adipose tissue (iWAT) and 2) whether overexpression of Lcn2 promotes whole-body metabolic homeostasis and healthspan. In the first project, we found Lcn2 deficient inguinal adipocytes have decreased expression of thermogenic genes and reduced mitochondrial capacity under basal conditions. Further, Lcn2 is necessary for retinoic acid (RA) induction of beiging markers in inguinal adipocytes, particularly in the presence of insulin. Lcn2 is required for insulin-stimulated localization of the retinoic acid receptor-alpha (RAR-α) to the plasma membrane. Together, this suggests Lcn2 promotes RA-induced thermogenesis, possibly through regulation of RAR-α at the plasma membrane. In the second project, we further investigated the role of Lcn2 in WAT beiging and energy metabolism *in vivo*. Utilizing an ap2-promoter-driven Lcn2 transgenic (Tg) mouse model, we determined whether overexpression of Lcn2 in adipose tissue is sufficient to promote beiging/thermogenesis. We found overexpression of Lcn2 in adipose tissue leads to improved cold adaptation and an increase in beiging markers in iWAT, including uncoupling protein 1 (*Ucp1*) gene expression. Lcn2 Tg mice had a trend towards

increased fat utilization, alongside increased oxidative gene expression and decreased adipocyte size in iWAT. In the third project, we investigated the role of Lcn2 in adipose tissue function and metabolic healthspan. We determined whether overexpression of Lcn2 in adipose tissue can prevent the age-associated decline in adipogenesis, serum metabolic parameters, and liver function. Following aging, Lcn2 Tg mice maintained higher levels of adipogenic markers and reduced adipocyte size in iWAT relative to WT mice, suggesting improved adipose tissue health. Aged Lcn2 Tg mice had decreased serum triglycerides, significantly better maintenance of glucose tolerance, and protection from liver lipid accumulation resulting in decreased markers of liver inflammation and steatosis. In conclusion, Lcn2 has a novel role in promoting WAT beiging and adipose tissue health, protecting against age-related metabolic deterioration, and improving metabolic healthspan.

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Literature Review

The high prevalence of obesity remains at the forefront of human health (1,2). Obesity predisposes individuals to diseases including type II diabetes, cardiovascular disease, and cancer (2,3). Obesity-associated diseases have widespread implications including decreased quality of life, decreased lifespan, and increased economic burden (3,4). Perturbations in adipose tissue function in response to obesity have been strongly associated with changes in whole body metabolism and the development of metabolic disease (5,6). As such, understanding the regulation of adipose tissue biology is an important step towards developing targeted interventions for obesity-related diseases.

I. White Adipose Tissue in Energy Metabolism

White Adipose Tissue Function

White adipose tissue (WAT) is contained within multiple depots, which are generally classified as visceral or subcutaneous WAT. Visceral adipose tissue, which surrounds the major organs in the abdomen, is thought to be more metabolically active then subcutaneous adipose tissue. Accumulation of fat in this depot is highly associated with the development of type II diabetes and metabolic disease (7). In addition to adipocytes, adipose tissue contains a diverse profile of immune cells, including macrophages, B cells, T cells, eosinophils and mast cells (8). These immune cells along with fibroblasts, endothelial cells and pre-adipocytes encompass the stromal vascular fraction (SVF) of adipose tissue.

The primary role of WAT is to serve as a depot to store excess glucose and fatty acids as triglyceride in the fed state (9). Adipose tissue directly takes up triglycerides from very low density lipoprotein (VLDL) as well as circulating free fatty acids. In response to insulin, adipose tissue can also take up glucose and perform de novo lipogenesis. During fasting, the rise in catecholamine levels results in the upregulation of hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), and monoglyceride lipase (MGL) in adipose tissue (10). These lipases hydrolyze triglycerides to free fatty acids and glycerol that are distributed to other tissues to meet energy requirements. The ability to store and mobilize lipids situates adipose tissue as a major regulator of whole-body energy homeostasis.

In addition to lipid storage, adipose tissue has been identified as an endocrine organ. Adipocytes have been found to secrete hundreds of hormones, cytokines and other signaling molecules termed adipokines (11). These secreted factors have both autocrine/paracrine effects as well as the ability to mediate cross-talk between WAT and distant tissues. Adipokines such as leptin, adiponectin, retinol binding protein 4, fibroblast growth factor 21 (FGF21), and lipocalin 2 (Lcn2) have a wide range of functions including regulating energy balance, metabolic homeostasis of glucose and lipids, insulin sensitivity and secretion, and immune response (12,13). In addition to adipocytes, resident immune cells also contribute to the secretory profile of WAT (14). Dysregulation of the secretory profile of adipose tissue is thought to be an underlying factor in the development of obesity and metabolic disease (13).

Adipose Tissue Developmental Origins

Adipocyte number increases during childhood before mostly stabilizing in adulthood, regardless of weight fluctuations (15). This indicates that in adulthood, adipose tissue growth results in increased fat cell size versus number. Despite stable fat cell number, carbon labeling studies have found that adipocytes turnover approximately every 10 years, with 10% of adipocytes being replaced every year (15). Therefore, studying the process of adipocyte development is relevant for understanding obesity prevention and treatment.

Adipocytes originate from adipose tissue stem cells (ASC) that are committed to preadipocytes in the SVF of adipose tissue (16,17). ASC are multipotent progenitor cells that can also differentiate to macrophages, osteoblasts, myocytes and many other cell types (16). Extracellular factors, including bone morphological proteins 2 and 4 (BMP2 and BMP4), promote the commitment of ASCs to pre-adipocytes (17). Insulin-like growth factor (IGF) and other nutrients and growth factors then promote the differentiation of pre-adipocytes to adipocytes. This activates a transcriptional cascade involving the CCAAT/enhancer-binding protein (C/EBP) family and perixosome proliferator-activated receptor γ (PPARγ) (17). The culmination of this is an increase in adipogenic genes that promote lipid droplet formation. Understanding the complexities of this process in the context of obesity and insulin resistance is an ongoing area of research.

White Adipose Tissue Dysfunction During Obesity and Metabolic Disease

In response to nutrient excess, WAT expands by increasing adipocyte number (hyperplasia) or size (hypertrophy). Storage of excess energy as lipids in adipocytes acts as a buffer to prevent circulating free-fatty acids from accumulating in non-adipose tissue such as liver, muscle, and heart (18). Ectopic accumulation of lipids contributes to the development of cardiovascular disease and type II diabetes, emphasizing the importance of WAT as a storage depot.

Despite this, excessive hypertrophy of adipocytes causes dysfunction, which promotes an inflammatory response in adipose tissue leading to disruption of whole-body metabolic homeostasis (5). Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) are secreted and are thought to mediate this response and contribute to the development of obesity-induced insulin resistance (19). Additionally, high-fat diet leading to increased adiposity and eventual adipocyte necrosis has been shown to promote macrophage infiltration of adipose tissue (20,21). In obese individuals, these adipose tissue macrophages (ATM) take on a more pro-inflammatory M1 phenotype, which is in opposition to their lean counterparts, who have more anti-inflammatory M2-like macrophages (20). These pro-inflammatory macrophages surround dead adipocytes to form crown like structures, where they may serve to absorb lipids released upon adipocyte death (21). Adipose tissue dysfunction is associated with or even causes insulin resistance, type 2 diabetes and other metabolic diseases. However, the causal relationship between adipose tissue inflammation and metabolic disturbances warrants further investigation.

While obesity is the leading cause of metabolic disease, attenuating the inflammatory response may be able to prevent these deleterious consequences. Mice lacking TNF-α function do not develop insulin resistance under obesigenic conditions, suggesting that attenuation of inflammation protects against metabolic disease (22). In addition, "metabolically healthy" obese individuals have decreased markers of inflammation in their adipose tissue when compared to their "metabolically unhealthy" counterparts (23). Therefore, the ability of adipocytes to mediate and resolve inflammation may hold the key to preventing the progression from obesity to metabolic disease.

II. Brown Adipose Tissue in Energy Metabolism

Brown Adipose Tissue Function

Traditionally, brown adipose tissue (BAT) is present in small, hibernating mammals and infants to produce heat in response to cold via adaptive non-shivering thermogenesis. In rodents, BAT is generally contained within one depot located in the interscapular area (24). More recently, positron emission tomography (PET) imaging studies utilizing radiolabeled glucose uptake as a marker for BAT have found depots in the neck, supraclavicular area, and around the vertebrae of adult humans (24-26). This has renewed interest in studying the function and regulation of BAT.

In contrast to WAT, BAT is more active in fatty acid oxidation and energy expenditure (27). BAT has a multilocular appearance with several, smaller lipid droplets,

which allows greater access for lipases to liberate fatty acids for oxidation. BAT features a higher mitochondrial content when compared to WAT, which supports increased oxidative capacity. Expression of the mitochondrial protein uncoupling protein 1 (UCP1) promotes the dissipation of the inner mitochondrial membrane proton gradient, resulting in the release of chemical energy as heat (27).

Differences in features of white and brown adipocytes can be attributed in part to a divergence in lineage. Unlike white adipocytes, brown adipocytes are derived from myogenic factor 5 expressing (*Myf5*+) progenitor cells (28). Skeletal muscle is also derived from *Myf5*+ cells, leading to similarity between brown adipose tissue and skeletal muscle transcriptomes and oxidative capacity (29).

In addition to its role in thermogenesis, several secreted factors with autocrine, paracrine, and endocrine functions have been identified in BAT (30,31). Although usually secreted from liver, FGF21 derived from BAT increases in serum in response to cold exposure (32). Though generally regarded as a pro-inflammatory cytokine, secretion of IL-6 from BAT has been shown necessary for BAT's improvement of insulin sensitivity (33). Neuregulin 4 (NRG4) is secreted from brown adipocytes and binds to hepatocytes, leading to downregulation of lipogenesis (34). Together, these and other factors suggest BAT can regulate metabolism through non-thermogenic mechanisms.

Regulation of BAT Thermogenesis

Classically, thermogenesis is activated by the sympathetic nervous system in response to cold exposure (35,36). Catecholamines released from neurons bind to the β3-

adrenergic receptor on the surface of brown adipocytes. This g-coupled receptor activates adenylyl cyclase, resulting in increased production of cyclic AMP (cAMP). This initiates a signaling cascade beginning with protein kinase A (PKA).

PKA phosphorylates the transcription factor cAMP response element binding protein (CREB), which induces transcription of the UCP1 gene (35,37). PKA has more recently been shown to phosphorylate p38 mitogen-activated protein kinase (p38MAPK), which in turn phosphorylates activating transcription factor 2 (ATF2) and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) (38,39). This results in increased transcription of the UCP1 and PGC-1α genes. Subsequent upregulation of PGC-1α drives mitochondrial biogenesis to support oxidation (40). Upregulation of UCP1 uncouples the electron transport chain from ATP synthase, leading to the dissipation of the inner mitochondrial membrane protein gradient. Additionally, PKA phosphorylates HSL, upregulating lipolysis to provide fatty acids for oxidation to fuel uncoupling (35). Fatty acids also directly bind to and are essential for activation of UCP1 protein (35).

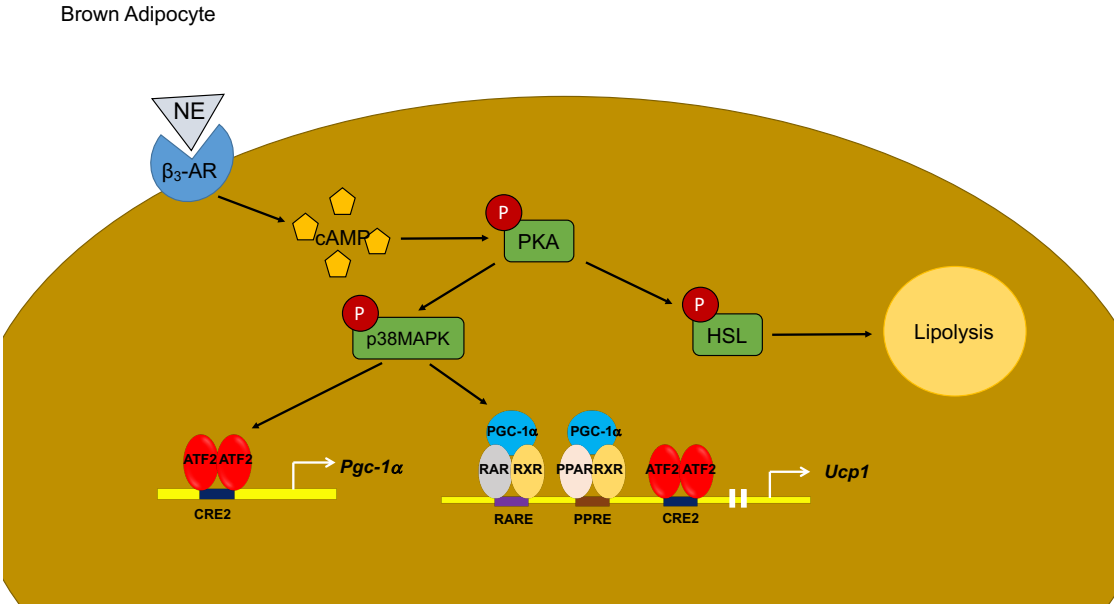

Figure 1: Regulation of BAT thermogenesis. Thermogenesis is classically activated in brown adipocytes by β-adrenergic signaling. The catecholamine norepinephrine (NE) binds to β_3-adrenergic receptors on the surface of adipocytes. This g-protein coupled receptor increases cAMP production, which phosphorylates PKA. PKA then phosphorylates p38MAPK, which phosphorylates the transcriptional regulators ATF2 and PGC-1α, resulting in transcription of thermogenic genes including *Ucp1* and *Pgc-1α*. Additionally, RAR-α, RXR, and PPARγ can also form heterodimers to regulate transcription of these genes. PKA also phosphorylates HSL, which facilitates lipolysis to provide fatty acids for

In addition to adrenergic agonists, thermogenesis can be upregulated in response to dietary factors. Diet-induced thermogenesis was first described in rats fed a higher fat, higher sugar, cafeteria diet (41,42). Following 2 weeks of cafeteria diet, rats had an increase in basal metabolic rate and increased BAT mass (42). This effect was not seen in insulin resistant rats, suggesting that insulin action is necessary for diet-induced thermogenesis (41). This has led to speculation that, evolutionarily, thermogenesis is increased in response to food intake to burn excess energy and maintain leanness. Despite this evidence, high-fat diet feeding in mice leads to downregulation of UCP1 in WAT,

leading to controversy over the concept of diet-induced thermogenesis as a means to prevent obesity (43).

Specific dietary components, including macro- and micronutrients, can also regulate thermogenesis. Intake of certain dietary lipids, including oleate-rich olive oil and omega-3 fatty acids, increases thermogenesis and oxidative metabolism (44). The vitamin A-derivative retinoic acid is a transcriptional regulator of UCP1 (45). The nuclear receptors retinoic acid receptor-alpha (RAR-α) and retinoid x receptor-alpha (RXR-α) can form heterodimers and bind to response elements in the enhancer region of the UCP1 gene (45-48). RA binds to these receptors and promotes translocation to the nucleus, resulting in enhanced transcription of UCP1 (45,49). More recent studies have found p38MAPK signaling is necessary for the RA's effect on UCP1 expression (50). This raises the possibility that either RA regulates thermogenesis through non-genomic mechanisms or an additional factor is required. The nuclear receptor PPARγ can also form a heterodimer with RXR and similarly bind to the enhancer region of the UCP1 gene (48). Therefore, PPARγ agonists including thiazolidinediones and fatty acid derivatives promote UCP1 transcription (51).

Endogenous circulating factors have also been identified as thermogenic activators. For example, cardiac naturietic peptides bind to receptors on adipocytes and increase p38MAPK signaling resulting in the upregulation of thermogenesis independent of β-adrenergic activation (52). The wide field of potential thermogenic activators requires further research to better understand how these molecules work together to regulate thermogenesis.

III. Beiging of White Adipose Tissue

Exposure to cold or adrenergic agonists leads to the development of brown-like adipocytes within WAT depots. These "beige" adipocytes are characterized by higher levels of UCP1, higher mitochondrial content, and a multilocular appearance more consistent with brown adipocytes. Beige adipocytes develop more readily in subcutaneous adipose tissue depots, such as inguinal WAT in mice, when compared to visceral adipose depots (53). These cells are thought to be molecularly distinct from brown adipocytes, as they do not arise from *Myf5*+ progenitor cells and express a different transcriptional signature then classic brown adipocytes (28,54). While beige adipocytes express both classical brown and white genes, beige specific genes have also been identified. These beige adipocyte markers include *Tbx1*, transmembrane protein 26 (*Tmem26*), and *CD137* (55).

There is controversy over whether beige adipocytes differentiate from precursor cells in WAT or whether they arise through conversion of pre-existing mature white adipocytes, termed "transdifferentiation". After cold exposure or treatment with adrenergic agonists, there is little proliferation of preadipocytes in WAT, supporting the idea that beige adipocytes are transdifferentiated from mature white adipocytes (56). On the other hand, a method that labels mature adipocytes prior to cold exposure has found that beige adipocytes develop from newly differentiated adipocytes as opposed to the labeled mature adipocytes (57). There is also evidence that, under thermoneutral conditions, previously recruited UCP1+ beige adipocytes look and behave like white

adipocytes until they are acutely activated again by cold or other thermogenic inducers (58). This suggests that it may be difficult to determine the difference between white adipocytes and beige adipocytes under thermoneutral conditions, adding further complexity to the issue. Therefore, the question of how beige adipocytes are recruited in WAT has not yet been resolved.

There has been further controversy over whether beige adipocytes are functional in regards to thermogenesis and whether they can significantly contribute to energy expenditure. Mitochondria isolated from inguinal WAT of cold-exposed mice are able to rapidly oxidize substrates in a manner similar to mitochondria from BAT, indicating beige adipocyte UCP1 is functional (59). When fully activated by forskolin, beige and brown adipocytes also express similar amounts of UCP1, further suggesting that beige adipose tissue is a major contributor to thermogenesis (55). Despite this, a majority of studies investigating beiging of WAT use molecular characteristics such as UCP1 gene expression and cellular oxygen consumption as markers of thermogenesis as opposed to measurement of bioenergetics and heat production (60). This makes it difficult to assess the true contribution of beige adipocytes to thermoregulation.

Adult humans have limited BAT, which makes the issue of recruiting beige adipose tissue in humans highly relevant if thermogenesis is to be used as a therapy for preventing obesity. Interestingly, recent studies analyzing biopsies of BAT from humans have found the molecular characteristics to be more similar to murine beige adipose tissue versus BAT (55). This indicates that adipose tissue depots in adult humans are adaptable and there could be potential to recruit beige adipose tissue. However, due to the obvious difficulties in obtaining biopsies, there are limited studies looking at beiging of

human WAT under inducible conditions. One human study looked at subcutaneous WAT from burn patients that have highly elevated levels of norepinephrine (61). WAT from burn patients expressed high levels of UCP1 and morphological changes indicated the recruitment of beige adipocytes when compared to control patients. This suggests that adult humans do have the ability to recruit beige adipose tissue, but it is unclear whether this ability is analogous to the beiging studies done in rodents.

Regulation of Beiging

Beige adipocytes can develop in WAT following prolonged cold exposure or treatment with adrenergic agonists (56,62). In addition to classical β-adrenergic activation, a variety of novel beiging agents have been identified.

Several studies have shown chronic treatment of cells with the anti-diabetic drug rosiglitazone can recruit beige adipocytes through its ability to act as a PPARγ agonist (53,54). This indicates that, similar to BAT, PPARγ plays a central role in the development of thermogenic adipocytes in WAT. In addition to PPARγ agonists, pharmaceutical agents that increase AMP-activated protein kinase (AMPK) and PPARα activity have been shown to increase UCP1 expression in WAT (44).

Endogenous secreted factors promote the development of beige adipocytes. In addition to regulating BAT thermogenesis, cardiac natriuretic peptides also induce beiging of WAT (52). FGF21 is increased during cold and post-transcriptionally regulates PGC-1α activity in WAT to induce thermogenic genes (63). In fact, FGF21 knock-out

mice have impaired induction of UCP1 in WAT in response to cold exposure (63), suggesting activation of additional, non-adrenergic pathways are necessary for beiging of WAT.

Several dietary metabolites can increase UCP1+ white adipocytes. Lactate and the ketone body β-hydroxybutyrate regulate the intracellular redox state, leading to increased thermogenic gene expression in white adipocytes (64). Similar to its action in BAT, the vitamin A-derivative RA can upregulate UCP1 gene expression in white adipose tissue (65,66). Recently, an increase in gut-derived short-chain fatty acids (SCFAs) following intermittent fasting has been shown to increase beiging of white adipose tissue in mice (67). Together, this suggests dietary composition and alterations in the metabolic environment can contribute to the beiging status of WAT.

In mice, wheel running leads to an increase in thermogenic genes in subcutaneous WAT, indicating exercise can increase beiging (68,69). Specific secreted factors from muscle, termed myokines, may regulate this. Irisin and IL-6 are secreted from muscle in response to PGC-1α overexpression or exercise and have been shown to induce beiging of WAT (69,70). However, it remains unclear whether there is an evolutionary purpose for exercise-induced beiging of WAT (71).

Thermogenesis: Obesity Prevention and Metabolic Health

Higher levels of BAT in humans is associated with a lower body mass index (BMI) and younger age (72). It is not yet clear whether increasing BMI is a cause or effect of downregulated BAT presence and activity. In mice, knock-out of UCP1 leads to

more weight gain versus control mice at thermoneutrality, indicating UCP1-mediated thermogenesis can prevent obesity (73). Recruitment of beige adipocytes by overexpression of PRDM16 in white adipose tissue of mice increased energy expenditure and prevented diet-induced obesity (74). This has led to interest in recruitment and activation of brown and beige adipocytes as a treatment for obesity in humans. Indeed, cold-stimulation or adrenergic agonist treatment in humans has been shown to increase energy expenditure in healthy individuals (75,76). Another study found treatment with a pan-adrenergic agonist led to increased BAT activity in lean, but not obese, individuals (77). In the obese individuals, it was unclear whether the impaired response was due to lack of BAT or impaired β-adrenergic signaling in BAT. If obese individuals do have impaired response to β-adrenergic agonists, this warrants further investigation of non-adrenergic mechanisms for increasing thermogenesis to prevent obesity.

In addition to increasing energy expenditure and protecting against obesity, BAT regulates substrate utilization to promote whole-body energy homeostasis. Transplantation of BAT into high-fat diet fed mice led to increased insulin-stimulated glucose uptake, improved glucose tolerance and increased insulin sensitivity (33). In humans, increased glucose disposal has been demonstrated following cold exposure or treatment with adrenergic agonists (78). By utilizing a hyperinsulinemia-euglycemia clamp, cold exposure has also been found to increase insulin sensitivity in humans, but only in those with detectable levels of BAT prior to being exposed to cold (79). Therefore, therapies that recruit brown and beige adipose tissue in humans are highly relevant for the treatment of insulin resistance.

Cold exposure also increases the expression of fatty acid transporters in BAT, resulting in increased fatty acid uptake in brown adipocytes and lowered plasma triglyceride and cholesterol levels (80). There is evidence that fatty acids taken up by brown adipocytes need to first be stored as intracellular triglycerides before being liberated by lipolysis and used as a substrate to fuel thermogenesis (81). This highlights the requirement of intact lipolytic activity to support thermogenesis.

Due to the ability of thermogenesis to clear plasma triglycerides, a role for BAT in the treatment and prevention of atherosclerosis has been recently investigated. Adrenergic activation of thermogenesis in genetically hyperlipidemic mice lowered plasma levels of cholesterol and triglycerides (82). Conversely, in *Ldlr* knock-out and *Apoe* knock-out mice prone to atherosclerosis, cold activation increased hypercholesterolemia and atherosclerotic plaque development (83). This suggests that in individuals prone to atherosclerosis, the increase in lipid mobilization during cold may have deleterious effects.

As inflammation in white adipose tissue is an underlying factor in the development of metabolic disease, it is relevant to ask whether increasing brown or beige activity can attenuate inflammation associated with obesity. One study found that genetically or pharmacologically inhibiting transient receptor pontential vanilloid 4 (TRPV4) increased beiging of WAT in lean mice (84). When challenged to a high-fat diet, mice with inhibited TRPV4 maintained higher levels of thermogenic genes in WAT resulting in significantly lower levels of proinflammatory cytokine expression (84). Therefore, recruitment of beige adipose tissue can prevent WAT inflammation that is

associated with excess caloric intake and may prevent the progression of obesity to metabolic disease.

IV. Adipose Tissue Regulates Aging and Healthspan

The average lifespan is increasing worldwide, with the number of individuals above the age of 65 set to rapidly increase in coming years (85). However, aging is a major risk factor for several diseases associated with obesity and metabolism, including diabetes, cardiovascular disease, and cancer (86-88). Several diseases of aging have also emerged in the face of increasing lifespan, most specifically neurodegenerative diseases (87). Therefore, finding ways to improve health and quality of life with aging, termed "healthspan", is a highly relevant area of research.

As humans age, there is an increase in adiposity and redistribution of fat stores in the body, positioning adipose tissue as a critical regulator of age-related pathologies. Subcutaneous adipose tissue decreases, while intra-abdominal, or visceral adipose tissue increases with aging (89,90). Resection of visceral adipose tissue from 20-month-old rats increases insulin sensitivity, indicating the accumulation of visceral fat with aging is a major contributor to shortened healthspan (91). Dysregulation of serum lipids results in ectopic accumulation of fat in the liver, heart, and muscle, which is associated with insulin resistance and cardiovascular disease (90,92). Increased body fat is not always accompanied by a change in body weight, due to loss of lean mass including bone and muscle with age (93,94). As muscle is the largest contributor to insulin-stimulated glucose uptake, this loss of lean mass further exacerbates glucose intolerance (93).

Together, the expansion and redistribution of adipose tissue is a major contributor to increased risk of metabolic disease with aging.

In addition to adipose tissue redistribution, aging is associated with changes in adipose tissue endocrine function. There is an increase in pro-inflammatory cytokines, including TNF-α and interleukin-6 (IL-6), produced in the adipose tissue of aged rodents (95,96). This increase in adipose-derived cytokines is thought to be a major contributor to aging-associated chronic, low-grade inflammation (96). The secretion of adipokines are also altered during aging. Serum leptin increases with age, independent of food intake or adiposity (97). However, older rats were less sensitive to the effects of externally administered leptin when compared to younger rats, suggesting leptin resistance develops with age (98). There have been contradicting reports on circulating adiponectin levels and aging (99,100). One study found that while there was no change in overall adiponectin levels in aged mice, the adiponectin/visceral fat ratio was decreased in 24-month-old mice when compared to younger mice (99). On the other hand, another study found higher plasma adiponectin levels in older women when compared to younger women, even after adjusting for increased visceral adiposity (100). Studies on centenarians have found higher serum adiponectin levels when compared to younger elderly individuals, suggesting high circulating adiponectin levels may play a role in longevity (101).

Several metabolic signaling pathways that are active in adipose tissue are involved in the pathology of aging. Decreased insulin-like growth factor 1 (IGF-1) and insulin pathway signaling has been associated with increased lifespan in several models of aging (102). IGF-1/insulin signaling negatively regulates several proteins, including forkhead box protein O1 (FOXO1) and the sirtuin family, that have been implicated in

longevity (102). Molecules that activate sirtuins, including resveratrol, have been shown to increase lifespan, insulin sensitivity, and mitochondrial function in high-fat diet fed mice (103). This was mediated in part through increased PGC-1α and AMP-activated protein kinase (AMPK) activity (103). Mammalian target of rapamycin (mTOR), which is downstream of insulin signaling, has also been linked to aging. Inhibiting mTOR, either through administration of rapamycin or genetically, has been shown to extend lifespan and protect against metabolic disease (104,105).

In addition to changes in WAT, BAT presence and thermogenic function are decreased in older adults when compared to younger adults (72,106,107). In mice, there is a dramatic decrease in UCP1 expression and beige adipocyte morphology in subcutaneous WAT from 12-month old mice when compared to 3-month old mice, indicating beige adipose tissue also declines with age (108). Further, aging mice deficient in UCP1 gained more weight on a high-fat diet when compared to WT aging mice (109). This suggests that age-related health decline could be prevented by therapies that increase adipose tissue thermogenic activity. Consequently, a recent study found that reversing senescence of beige progenitor cells led to increased induction of beiging and improved glucose sensitivity in aging mice and humans (110).

V. Lipocalin 2

Lipocalin 2: Expression and Regulation

Lipocalin 2 (Lcn2), or neutrophil gelatinase-associated lipocalin, is a 25 kD secreted protein first characterized in neutrophils (111). In addition to neutrophils and other immune cells, Lcn2 is also expressed in a range of tissues including bone marrow, uterus, prostate, stomach, lung and colon (112). Solution structures of Lcn2 indicate it has an anti-parallel β-barrel structure, which surrounds a hydrophobic binding pocket (113). This structure is conserved among the lipocalin family and allows them to bind to a variety of small, hydrophobic molecules such as fatty acids, retinoids, and steroids (114). More recent crystal structures have found the binding pocket of Lcn2 is larger and contains a higher number of polar and positive residues when compared to other members of the lipocalin family (115). In addition to its ability to bind lipids, the 178-amino acid protein can be glycosylated on an Asparagine positioned at residue 65 and the human homolog contains an unpaired cysteine located at residue 87, which enables dimerization with other proteins (111,113). The promoter region of the Lcn2 gene contains binding motifs for nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and CCAAT enhancer binding protein (C/EBP), as well as several nuclear receptor response elements including glucocorticoid response element, estrogen response element and retinoic acid receptor response element (116-118). Together, these features suggest a diversity of functions in the cell.

Lcn2 was originally found to bind to and stabilize matrix-metalloproteinase-9 (MMP-9), which is involved in extracellular matrix remodeling (111,119). Later, activation of toll-like receptors (TLRs) on the surface of immune cells by LPS was found to enhance transcription and secretion of Lcn2 (120,121). This led to a well-characterized role in the innate immune system, where it has been shown to inhibit bacterial growth by

sequestering iron-containing siderophores (121,122). As such, Lcn2 deficient mice are more susceptible to bacterial infections (123). In this way, Lcn2 is upregulated by inflammatory stimuli but plays a protective role in the body's response to infection.

Lcn2 is a secreted protein, which has led to interest in potential Lcn2 receptors and binding partners. Lcn2 was first shown to bind to the cell surface receptor megalin, which results in the endocytosis of both apo-Lcn2 and siderophore-bound Lcn2 (124). The high affinity of Lcn2 for megalin is thought to relate to the role of Lcn2 in iron metabolism. A specific Lcn2 receptor, LCN2-R or SLC22A17, has also been identified but the role this receptor plays in regulation of cellular processes is controversial and poorly characterized (125). One study has shown that Lcn2 bound to iron-containing siderophores can bind to the LCN2 receptor and be endocytosed to increase intracellular iron content (126). Another study found that Lcn2 has a weak affinity for the LCN2 receptor and cast doubt on the ability of Lcn2 to transport iron into cells (127). In addition to the Lcn2 receptor and megalin, Lcn2 has been shown to bind directly to phosphatidylcholine in lipid rafts of the plasma membrane (128). This has led to speculation that Lcn2 can facilitate lipid raft reorganization to promote cell signaling events (129).

Lipocalin 2 and Adipose Tissue Metabolism

There is contention over the role Lcn2 plays in the regulation of metabolic disease as multiple groups have reported divergent phenotypes. Our lab has found that Lcn2 plays an important role in the regulation of thermogenesis, insulin resistance,

dyslipidemia and fatty liver disease (130-132). We have shown that Lcn2 knock-out (KO) mice fed a high-fat diet (HFD) consisting of 65% lard display worsened obesity, adipocyte hypertrophy, insulin resistance and fatty liver when compared to WT mice (131). On the other hand, Wang group used a different knock-out approach and found that while Lcn2 KO mice have fat mass gain and adipocyte hypertrophy on a HFD containing 45% soy oil, the KO mice are more insulin sensitive when compared to WT mice (133). A third study by Rosen group has found no significant changes in metabolic phenotype when comparing Lcn2 KO mice to WT mice on a HFD containing 45% soy oil (134). Differences in the knock-out approach, as well as variances in HFD consistency, may contribute to the conflicting phenotypes seen in these studies. A recent study found that Lcn2 secreted from bone promotes satiety and improves glucose sensitivity (135). These findings support the idea that Lcn2 can promote metabolic health and prevent obesity-associated diseases.

Lcn2 has previously been shown to activate M2 macrophage polarization, which is thought to be anti-inflammatory when compared to the pro-inflammatory M1 phenotype (136). This, in addition to its regulation by inflammatory cytokines, suggests a role for Lcn2 in obesity-related inflammation (137). Consistent with this, our lab has found that M2 markers are downregulated and M1 markers are upregulated in adipose tissue and liver from $Lcn2^{-/-}$ mice fed a HFD (138). Bone-marrow derived macrophages (BMDM) from $Lcn2^{-/-}$ mice have a more pro-inflammatory response after LPS treatment when compared to WT BMDMs (138). Additionally, treatment of 3T3-L1 adipocytes with Lcn2 attenuates the response to TNF-α and addition of Lcn2 to macrophages

decreases cytokine production in response to LPS (130). This indicates that Lcn2 may help to resolve the deleterious effects of overnutrition and inflammation in adipose tissue.

Lcn2 expression and protein level is increased in response to thermogenic stimuli, including cold exposure and treatment with the catecholamine norepinephrine (137). When placed in a cold environment, Lcn2 KO mice were unable to maintain their body temperature, indicating impaired non-shivering thermogenesis (131). Further studies found that Lcn2 deficient mice have impaired thermogenic programming in BAT (139). While Lcn2 deficient mice have a normal response to catecholamines in BAT, p38MAPK signaling is impaired indicating a non-adrenergic mechanism by which Lcn2 regulates thermogenesis. Exploration of alternative mechanisms of thermogenic regulation found that Lcn2 mediates the effect of retinoic acid (RA) on thermogenic programming in BAT (140). While it is clear that Lcn2 regulates BAT thermogenesis, whether and how Lcn2 regulates thermogenesis in WAT has not been well studied.

VI. Current Objectives

Our lab has previously shown that mice deficient in Lcn2 have impaired BAT thermogenesis (139). However, adult humans have limited stores of BAT, especially older or obese adults that are more susceptible to metabolic disease (72,106,107). As thermogenic activity improves metabolic homeostasis (33,75,79), therapies that increase recruitment and activity of beige adipocytes in WAT may hold more promise for preventing obesity-related diseases. In the second chapter of this thesis, we sought to determine whether Lcn2 is necessary for the recruitment and activation of beige

adipocytes in WAT. We also investigated a potential mechanism by which Lcn2 regulates beiging.

There is controversy over whether increased Lcn2 in response to obesity and inflammation has a protective effect against metabolic disease (130-134). To date, all studies have been conducted in whole-body $Lcn2^{-/-}$ mice. The effect of overexpressing Lcn2, particularly adipose tissue Lcn2, on thermogenesis and the prevention of obesity-related disease is unknown. Therefore, in the third chapter of this thesis we investigated the effect of increasing endogenous Lcn2 expression in adipose tissue by generating ap2-promoter-driven Lcn2 transgenic mice. We determined whether overexpression of Lcn2 in adipose tissue improves cold tolerance, energy expenditure, adiposity, diet-induced obesity, glucose homeostasis, and serum lipids.

Lastly, aging is a major risk factor for the development of obesity and metabolic disease (86,88). Aging is associated with increased adiposity, insulin resistance, dyslipidemia, decreased brown and beige adipose tissue thermogenesis and inflammation (89-92,95,96,108). Circulating levels of adipokines, including leptin and adiponectin, are altered during aging and may play a role in longevity and healthspan (91,97,100). Young mice deficient in the adipokine Lcn2 have a worsened metabolic profile (131,132,139), indicating Lcn2 may protect against metabolic disease. However, how Lcn2 levels change with aging and the effect of chronically overexpressing Lcn2 in adipose tissue during aging has not been well studied. In the fourth chapter of this thesis, we aged male and female Lcn2 transgenic mice to investigate whether overexpressing Lcn2 in adipose tissue has a protective effect during aging. We sought to determine how Lcn2 levels

change during aging and the effect of Lcn2 overexpression on age-related changes in adiposity, glucose homeostasis, dyslipidemia, liver steatosis, and inflammation.

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Chapter 2

Lipocalin 2 Regulates Retinoic Acid-Induced Beiging of White Adipocytes

Abstract

Lipocalin 2 (Lcn2) has previously been characterized as an adipokine regulating thermogenic activation of brown adipose tissue and retinoic acid (RA)-induced thermogenesis in mice. The objective of this study was to explore the role and mechanism for Lcn2 in the recruitment and retinoic acid-induced activation of brown-like or "beige" adipocytes. We found Lcn2 deficiency reduces key markers of thermogenesis including uncoupling protein-1 (UCP1) and peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) in inguinal white adipose tissue (iWAT) and inguinal adipocytes derived from $Lcn2^{-/-}$ mice. $Lcn2^{-/-}$ inguinal adipocytes have impaired p38 mitogen-activated protein kinase (p38MAPK) signaling pathway activation. This is accompanied by a lower basal and maximal oxidative capacity in $Lcn2^{-/-}$ inguinal adipocytes, indicating reduced mitochondrial capacity. On the other hand, recombinant Lcn2 induces p38MAPK phosphorylation and thermogenic gene expression in both wild-type (WT) and $Lcn2^{-/-}$ inguinal adipocytes. Rosiglitazone treatment during differentiation of $Lcn2^{-/-}$ adipocytes is able to recruit beige adipocytes at a normal level, however further activation of beige adipocytes by insulin and RA is impaired in the absence of Lcn2. Further, the synergistic effect of insulin and RA on UCP1 and PGC-1α expression is markedly reduced in $Lcn2^{-/-}$ inguinal adipocytes. Most intriguingly, Lcn2 and the retinoic acid receptor-alpha (RAR-α) are concurrently translocated to the plasma membrane of adipocytes in response to insulin, and this insulin-induced RAR-α translocation is absent in adipocytes deficient in Lcn2. Our data indicates a model where Lcn2 regulates RA-induced activation of beige adipocytes by promoting insulin-stimulated localization of

RAR-α to the plasma membrane. This suggests a novel Lcn2-mediated pathway by which insulin regulates the non-genomic actions of RA on thermogenic signaling activation.

Introduction

Obesity prevalence continues to increase along with escalating rates of metabolic diseases including diabetes and cardiovascular disease (1). Thermogenic activity in adipose tissue is associated with a decreased BMI and improved metabolic health (2,3). Although the presence of brown adipose tissue (BAT) in adult humans has been confirmed, the BAT established in infancy dramatically decreases with age (4). Therefore, the conversion of energy storing white adipose tissue (WAT) to energy expending BAT holds therapeutic potential. White adipocytes can take on a brown-like, or "beige" phenotype, characterized by increased number of mitochondria, a multilocular appearance, expression of uncoupling protein-1 (UCP1) positive cells, and a functional increase in energy expenditure (2,5).

Several factors are known to promote beiging of WAT, including cold, dietary factors, exercise, pharmaceuticals and circulating factors including adipokines (2). Thermogenesis is classically activated by catecholamines released during cold exposure, which bind to β-adrenergic receptors on the surface of adipocytes. This activates a protein kinase A, p38 mitogen-activated protein kinase (p38MAPK), activating transcription factor-2 (ATF2) signaling cascade which concurrently upregulates mitochondrial biogenesis, the expression of thermogenic genes, and lipolysis to provide fatty acids to support uncoupling of the electron transport chain (6,7). However, alternative, non-adrenergic pathways have also been identified as promoting beiging of WAT.

The anti-diabetic drug rosiglitazone acts as a peroxisome proliferator-activated receptor γ (PPARγ) agonist and upregulates transcription of mitochondrial and thermogenic genes including *Ucp1* (5,8,9). Chronic treatment of white adipose tissue

with rosiglitazone recruits beige adipocytes, but is not by itself able to activate thermogenesis without further adrenergic stimulation (10). It is unclear whether endogenous factors exist that act in a similar manner to rosiglitazone, as well as whether non-adrenergic beiging activators require or influence PPARγ-mediated recruitment of beige adipocytes.

Retinoic acid (RA), a known activator of BAT thermogenesis, binds to and promotes nuclear translocation of transcription factors that upregulate *Ucp1* gene expression (11-14). RA is also reported to increase p38MAPK phosphorylation, which was found necessary for the RA-induced upregulation of *Ucp1* gene expression (15,16). Therefore, it is possible that RA regulates thermogenesis through multiple pathways, although the exact mechanism is unknown.

Lipocalin 2 (Lcn2) is a 25 kDa secreted protein expressed highly in adipose tissue depots in response to cold and inflammatory stimulation (17,18). Solution structures of Lcn2 indicate it has an anti-parallel β-barrel structure, which surrounds a hydrophobic binding pocket (19). This structure is conserved among the lipocalin family and allows them to bind to a variety of small, hydrophobic molecules such as fatty acids, retinoids, and steroids (20). It has previously been shown that $Lcn2^{-/-}$ mice are not able to maintain their body temperature when exposed to cold (18). Subsequent examination of BAT in $Lcn2^{-/-}$ mice found deficiencies in induction of thermogenic genes and mitochondrial biogenesis following cold exposure (21). Conversely, catecholamine levels and the response to β-adrenergic activators are normal in $Lcn2^{-/-}$ mice and brown adipocytes, suggesting Lcn2 regulates thermogenesis through a non-adrenergic mechanism (21). $Lcn2^{-/-}$ mice have decreased expression of PPARγ in brown and white adipose tissue

depots and chronic treatment with rosiglitazone has been shown to improve insulin sensitivity and cold intolerance in these mice (22). Further studies have found Lcn2 regulates RA metabolism and action in adipose tissue (23). These previous studies suggest that Lcn2 may regulate RA-induced thermogenesis in adipose tissue. However, the exact pathway by which Lcn2 regulates adipose tissue, especially white adipose tissue, thermogenesis remains unknown. Therefore, the objective of this study was to determine the role of Lcn2 in the regulation of beiging of white adipocytes and to identify a non-adrenergic pathway that is responsible for Lcn2 regulation of RA-induced activation of beige adipocytes.

Materials and Methods

Animals

Lcn2-deficient mice were kindly provided by Dr. Alan Aderem, Institute for Systems Biology, Seattle, Washington, USA. Heterozygous mating scheme was used to generate wild-type (WT) and $Lcn2^{-/-}$ mice as previously described (18). Animals were housed at 22°C in a specific pathogen-free facility at the University of Minnesota. Animal studies were conducted with the approval of the University of Minnesota Animal Care and Use Committee and conformed to the National Institute of Health guidelines for laboratory animal care.

For *in vivo* studies with retinoic acid treatment, WT and $Lcn2^{-/-}$ mice from the same litter were housed at 22°C in a 12:12-h light-dark cycle with free access to water. Male mice were fed a high fat diet (HFD) (60% of calories from fat, Bio-serv F3282, New Brunswick, NJ) at four weeks of age for 12 weeks. At 16 weeks of age, 3 male WT

and 5 male Lcn2$^{-/-}$ mice were treated with 50 mg/kg body weight all-trans retinoic acid (ATRA, Sigma, St. Louis, MO) via oral gavage daily for 24 days. Control mice were treated with 1% ethanol vehicle. HFD was continued during the 24-day treatment period. After 24-day treatment, tissues were collected and immediately frozen in liquid nitrogen and stored at -80°C for use.

Cell culture and differentiation of primary stromal-vascular cells

Stromal-vascular (SV) cell fraction containing pre-adipocytes were isolated from inguinal WAT of WT and Lcn2$^{-/-}$ male mice as previously described (21). Cells were grown to confluence in Dubecco's Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY) containing L-glutamine and 25 mM glucose, 10% FBS (Atlanta Biological, Lawrenceville, GA) and 100 units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA). After reaching confluence, pre-adipocytes were treated with a differentiation cocktail for 2 days containing DMEM, 10% FBS, penicillin/streptomycin, 0.25 mM isobutylmethylxanthine (Sigma, St. Louis, MO), 0.5 μM dexamethasone (Sigma, St. Louis, MO), and 0.85 μM insulin. The cells were then maintained in culture media with 0.85 μM insulin until fully differentiated. For rosiglitazone experiments, 1 μM rosiglitazone (Sigma, St. Louis, MO) was added to the culture media from day 0 of differentiation until fully differentiated. For insulin stimulation, differentiated adipocytes were serum starved in DMEM containing 0.5% FBS for 3-6 hours prior to addition of insulin. Recombinant Lcn2 was commercially obtained from R&D Systems (Minneapolis, MN).

Cellular respiratory assay

Cellular oxygen consumption rate (OCR) was analyzed using the XF24 Analyzer (Seahorse Bioscience, Billerica, MA). Inguinal stromal-vascular cells were differentiated in XF24 V7 culture plates. After differentiation, cellular oxygen consumption was measured three times under basal conditions and three times after addition of each of the following: 2 μM oligomycin, 0.5 μM FCCP, and 4 μM antimycin. OCR (pmol/min) was normalized to the untreated control.

NAD+/NADH Assay

Levels of intracellular NAD+ and NADH in differentiated inguinal adipocytes were measured using the NAD/NADH assay kit from Sigma (St. Louis, MO) according to the manufacturer's instructions. On day 7 of differentiation, inguinal adipocytes were washed twice in PBS and harvested in 500 μL of extraction buffer. Following centrifugation, half of the supernatant was heated at 60°C for 30 minutes to degrade NAD for determination of NADH levels. Native and heated samples were added to a 96-well plate and colorimetrically measured. Total NAD and NADH levels were calculated by comparison to a standard curve and ratio of NAD/NADH was calculated by the equation (NAD-NADH/NADH).

Mitochondrial DNA content

Differentiated inguinal adipocytes were lysed in a buffer containing Tris-HCL, EDTA, and SDS and incubated at 55°C overnight. Chloroform and potassium acetate were added to the lysis buffer and centrifuged at 10°C at 13,000 rpms for 5 minutes. The

supernatant was collected and isopropanol was added prior to centrifuging at 10°C at 10,000 rpms for 10 minutes. The resulting pellet was washed twice with 75% ethanol and centrifuged at 10°C at 7400 rpms for 5 minutes after each wash. The final pellet containing DNA was reconstituted in DNase free water and adjusted to equivalent quantities. CoxII was used as a mitochondrial DNA marker and normalized to the nuclear DNA marker Rip140.

Plasma membrane isolation

For the preparation of crude plasma membrane fractions, a previously described method was followed (24). Briefly, differentiated primary adipocytes were harvested and homogenized in TES buffer containing 20 mM Tris, 1 mM EDTA, and sucrose. Homogenates were layered onto a sucrose cushion and centrifuged at 28,000 rpm for 20 minutes in a SW60 Ti swinging bucket rotor. Interface was collected and centrifuged at 55,000 rpm for 10 minutes in a TLA120.2 rotor to obtain the final pellet containing the plasma membrane fraction.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from inguinal WAT or inguinal primary adipocytes with TRIZOL reagent (Invitrogen, Carlsbad, CA). RNA was DNase-treated prior to synthesis of cDNA using a Superscript II reverse transcription kit (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was conducted using SYBR Green qPCR Master Mix (SABiosciences, Frederick, MD) with a StepOne Real-Time PCR System (Applied

Biosystem, Foster City, CA). The ΔΔCt method was used to calculate mRNA expression. β-Actin or Tbp served as an internal control.

Western blot analysis

Inguinal WAT or differentiated inguinal adipocytes were homogenized in RIPA buffer (Sigma, St. Louis, MO). Protein was quantified using the bicinchoninic acid method (Pierce, Rockford, IL). Equivalent amounts of protein were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane prior to incubation with primary and secondary antibodies. All primary antibodies were obtained from Cell Signaling Technology (Danvers, MA) except for mouse monoclonal UCP-1 antibody from R&D Systems (Minneapolis, MN). Antibodies included β-Actin: Cat#4967L RRID: AB_330288, phospho p38MAPK: Cat#9211S RRID: AB_331641; total p38MAPK Cat#9212S RRID: AB_330713; phospho HSL: Cat#4139S RRID: AB_2135495; total HSL: Cat#4107 RRID: AB_2296900; UCP1: Cat#MAB6158 RRID: AB_10572490; PPARγ: Cat#2435S RRID: AB_2166051; phospho Akt (ser473): Cat#9271S RRID: AB_329825; phospho Akt (thr308): Cat#9275S RRID_AB329828; phospho p70 s6k: Cat#9205S RRID: AB_330944; total Akt: Cat#9272S RRID: AB_329827; Lcn2: Cat#AF1857 RRID: AB_355022; RARα: Cat#2554S RRID: AB_10696877; Na+/K+ ATPase: Cat#3010S RRID: AB_2060983. Anti-rabbit secondary antibody and anti-mouse secondary antibody are from R&D Systems (Minneapolis, MN) and used at a dilution of 1:5000. ECL western blotting substrate (Pierce, Rockford, IL) was used to detect reactivity.

Statistical analysis

Values are reported as mean +/- standard error of the mean (SEM). Statistical significance was determined by two-tailed Student's *t* test, where a *P* value less than 0.05 was considered significant.

Results

1. *Lcn2 deficiency impairs beiging and mitochondrial function in inguinal white adipose tissue.*

Lcn2 gene expression and protein levels in inguinal white adipose tissue (iWAT) are increased in response to thermogenic stimuli, including cold exposure and treatment with the β-adrenergic agonist norepinephrine (NE) (17,18). We therefore sought to determine a role for Lcn2 in WAT beiging. We found *Ucp1* expression was significantly attenuated in iWAT from $Lcn2^{-/-}$ mice, signifying possible impairments in beiging in the absence of Lcn2 (Fig. 1A). This effect was not seen in epididymal white adipose tissue (eWAT), which has previously been reported to have less thermogenic capacity than iWAT (25). Further, primary adipocytes isolated from iWAT from $Lcn2^{-/-}$ mice showed significantly attenuated expression of thermogenic genes *Ucp1* and peroxisome proliferator-activated receptor gamma coactivator 1α (*Pgc-1α*) compared to those from WT mice (Fig. 1B), suggesting impairment in beiging capacity.

Since beiging capacity is positively correlated with mitochondrial function (26), we next looked at functional measurements of mitochondrial activity. Mitochondrial respiratory profile analysis by Seahorse XF Analyzers showed that oxygen consumption rates were significantly decreased in $Lcn2^{-/-}$ differentiated inguinal adipocytes under both

basal and maximal respiration conditions, indicating a decrease in mitochondrial oxidative capacity (Fig. 1C, 1D). Consistent with decreased mitochondrial function, NAD/NADH+ ratio was decreased in $Lcn2^{-/-}$ inguinal adipocytes under both fed and starved conditions (Fig. 1E). Mitochondrial DNA copy number was similar between WT and $Lcn2^{-/-}$ inguinal adipocytes, indicating dysfunction of mitochondria in $Lcn2^{-/-}$ inguinal adipocytes versus a reduction in mitochondrial content (Fig. 1F). Taken together, this data suggests adipocytes deficient in Lcn2 have reduced beiging capacity and impaired mitochondrial function.

2. *Lcn2 regulates p38MAPK signaling pathway activation and thermogenic gene expression in inguinal adipocytes.*

As p38MAPK signaling is the primary upstream pathway regulating thermogenic gene expression (6,7), we next investigated whether Lcn2 regulates this thermogenic pathway in inguinal adipocytes. Stromal-vascular cells isolated from WT and $Lcn2^{-/-}$ iWAT were differentiated to mature adipocytes. Differentiated adipocytes were treated with recombinant Lcn2 in the absence or presence of insulin for one hour or 18 hours to examine the acute and chronic effects of Lcn2 on p38MAPK signaling. There was no statistically significant difference in basal levels of p38MAPK phosphorylation between WT and $Lcn2^{-/-}$ adipocytes (Fig.2A, 2B). After one hour of treatment, insulin did not seem to stimulate phosphorylation of p38MAPK (Fig 2A, 2B). However, one-hour treatment with recombinant Lcn2 significantly increased phosphorylation of p38MAPK in both the presence and absence of insulin in WT adipocytes. After 18-hour treatment, insulin significantly increased p38MAPK phosphorylation in WT adipocytes, but the

effect of insulin was blunted in Lcn2-/- adipocytes (Fig. 2C, 2D). Treatment with recombinant Lcn2 for 18 hours was able to restore the effect of insulin on phosphorylation of p38MAPK in Lcn2$^{-/-}$ adipocytes (Fig. 2C, 2D). Similar to 18-hour insulin-stimulated p38MAPK phosphorylation, insulin increased *Ucp1*, *Pgc1-α,* and *Cidea* gene expression in WT adipocytes, but not Lcn2$^{-/-}$ adipocytes (Fig. 2E). Treatment with recombinant Lcn2 was able to significantly increase *Ucp1* and *Pgc1-α* gene expression in Lcn2$^{-/-}$ adipocytes (Fig 2F). Together, this data suggests that Lcn2 is required for and can increase insulin-stimulated p38MAPK phosphorylation and expression of thermogenic genes.

3. Lcn2 is not necessary for β-adrenergic signaling in inguinal adipocytes.

To test the impact of Lcn2 deficiency on β-adrenergic signaling in adipocytes, we treated differentiated inguinal adipocytes from WT and Lcn2$^{-/-}$ mice with norepinephrine (NE) for one hour. Phosphorylation of p38MAPK in response to acute NE treatment was higher in Lcn2$^{-/-}$ adipocytes when compared to WT, indicating β-adrenergic signaling is intact in the absence of Lcn2 (Fig. 3A). Similarly, there was no significant difference in induction of *Ucp1* gene expression by NE in Lcn2$^{-/-}$ differentiated inguinal adipocytes when compared to WT (Fig. 3D). As insulin-induced thermogenic signaling and gene expression is impaired in the absence of Lcn2, we also tested β-adrenergic signaling in the presence of insulin. However, NE-induced phosphorylation of p38MAPK remained higher in Lcn2$^{-/-}$ adipocytes despite the addition of insulin (Fig. 3A). Similar to p38MAPK, phosphorylation of hormone sensitive lipase (HSL) in response to acute NE

treatment was higher in Lcn2$^{-/-}$ inguinal adipocytes when compared to WT adipocytes (Fig.3B). Next, we treated differentiated inguinal adipocytes with isoproterenol and measured glycerol release as an indicator of β-adrenergically activated lipolysis. Isoproterenol was able to induce glycerol release in Lcn2$^{-/-}$ inguinal adipocytes, although this was slightly decreased when compared to WT inguinal adipocytes (Fig. 3C). Together, this data points towards a non-adrenergic mechanism by which Lcn2 regulates thermogenic signaling in inguinal WAT.

4. *Lcn2 deficiency impairs retinoic acid-induced beiging and thermogenesis in Lcn2$^{-/-}$ inguinal adipocytes.*

Retinoic acid (RA) has previously been shown to increase UCP1 expression, lipolysis, mitochondrial function, and brown-like morphology of WAT adipocytes (11,13,27). RA is known to exert its effect on thermogenesis through binding and nuclear translocation of the retinoic acid receptor-alpha (RAR-α), which serves as a transcription factor for several genes including *Ucp1* and *Pgc-1α* (11,14,15). It has previously been reported that the lipocalin family of proteins bind to hydrophobic ligands including retinoids (20), so we next sought to determine whether Lcn2 is involved in RA-induced beiging of WAT. As shown in Fig. 4A, 4B, and 4C chronic treatment (24 days) of 12-week HFD fed mice with RA resulted in a significant increase in UCP1 protein levels and thermogenic gene expression in iWAT of WT mice, but this effect was completely ameliorated in inguinal WAT from Lcn2$^{-/-}$ mice. In *in vitro* studies with differentiated inguinal adipocytes, acute (24h) treatment of WT inguinal adipocytes with RA resulted in an upregulation of *Ucp1* gene expression, but this was blunted in adipocytes from Lcn2$^{-/-}$

mice (Fig. 4D). When WT inguinal adipocytes were treated with RA in the presence of insulin, a synergistic effect was observed on *Ucp1* and *Pgc-1α* gene expression (Fig. 4D, 4E), suggesting that RA requires insulin for maximal activation of thermogenic gene expression in white adipocytes. The synergistic effect between RA and insulin on gene expression was significantly attenuated in $Lcn2^{-/-}$ inguinal adipocytes. Interestingly, RA was able to rapidly phosphorylate p38MAPK in WT inguinal adipocytes, indicating that RA may have a non-genomic effect on thermogenic signaling (Fig. 4F, 4G). This rapid effect of RA on p38MAPK phosphorylation was abolished in $Lcn2^{-/-}$ inguinal adipocytes. This data indicates that Lcn2 is necessary for RA induction of thermogenic gene expression and UCP1 protein levels in inguinal white adipocytes, and may regulate this by promoting the rapid phosphorylation of p38MAPK by RA.

5. *Lcn2 deficiency impairs activation, but not recruitment, of beige adipocytes in Lcn2-/- inguinal adipocytes.*

While $Lcn2^{-/-}$ inguinal adipocytes have reduced thermogenic signaling and gene expression in response to insulin and RA, it is possible that impaired recruitment of UCP1+ beige adipocytes is driving the decrease in thermogenic activation induced by RA in $Lcn2^{-/-}$ mice. The transcription factor PPARγ has previously been shown to be involved in brown adipocyte differentiation and beiging of WAT (5,9). In WT and $Lcn2^{-/-}$ inguinal adipocytes differentiated with or without rosiglitazone, we found similar levels of lipid accumulation which indicates equivalent rates of differentiation (Fig. 5A). Further, expression of adipogenic genes and PPARγ protein levels were similar between WT and $Lcn2^{-/-}$ adipocytes (Fig. 5B, 5D). To test the effect of rosiglitazone on recruitment of

beige adipocytes, we next treated WT and Lcn2$^{-/-}$ adipocytes with rosiglitazone during differentiation followed by an acute stimulation of the fully differentiated adipocytes with RA. Treatment of inguinal adipocytes with rosiglitazone during differentiation results in similar *Ucp1* gene expression in WT and Lcn2$^{-/-}$ inguinal adipocytes indicating normal recruitment of beige adipocytes in response to PPARγ activation (Fig. 5C). However, rosiglitazone during differentiation was not able to rescue the acute effect of RA on *Ucp1* gene expression. Likewise, rosiglitazone during differentiation induces similar levels of UCP1 protein in WT and Lcn2$^{-/-}$ inguinal adipocytes, while the acute addition of RA for 24 hours results in a further marked increase in UCP1 protein levels in WT, but this increase was attenuated in Lcn2$^{-/-}$ adipocytes (Fig. 5D). This indicates that while the recruitment of beige adipocytes and thermogenic capacity by rosiglitazone is intact, thermogenic activation of beige adipocytes by RA is impaired in Lcn2$^{-/-}$ inguinal adipocytes.

We saw a synergistic effect between RA and insulin on thermogenic gene expression, leading us to question whether the insulin signaling pathway is involved in regulation of thermogenic activation. Indeed, activation of mammalian target of rapamycin complex 1 (mTORC1) has recently been found necessary for β-adrenergic beiging of white adipose tissue (28). Therefore, we hypothesized mTORC1 activation by insulin may be required to fully activate RA-induced beiging and that defects in insulin signaling in Lcn2$^{-/-}$ inguinal adipocytes could explain the differences in response to insulin and RA in these cells. Inguinal SV cells from WT and Lcn2$^{-/-}$ mice were differentiated to adipocytes with rosiglitazone, followed by 30-minute treatment with RA in the presence or absence of insulin. However, there were no differences in

phosphorylation of Akt or ribosomal protein S6 kinase (S6K) between WT and Lcn2$^{-/-}$ rosiglitazone-recruited beige adipocytes in response to 30-minute insulin stimulation (Fig. 5D). Further, 30-minute RA treatment did not increase phosphorylation of S6K with or without the presence of insulin, suggesting mTORC1 activity does not mediate the effects of RA on beiging. This suggests Lcn2 regulates the synergistic effect of insulin and RA on thermogenic gene expression through a mechanism independent from the insulin-mTORC1 signaling pathway.

6. Lcn2 is required for insulin-induced RAR-a translocation to the plasma membrane.

As we saw rapid phosphorylation of p38MAPK by RA, we next pursued non-genomic mechanisms by which RA and insulin could synergistically regulate thermogenesis. A pool of RAR-α in plasma membrane lipid rafts has previously been reported to facilitate RA action on p38MAPK in mouse embryonic fibroblasts (29,30). Lcn2 is a secreted protein which has been shown to localize to the plasma membrane and has been speculated to be involved in lipid raft rearrangement (31,32). Additionally, our group has previously shown normal translocation of RAR-α to the nucleus and normal induction of RA-responsive non-thermogenic genes in Lcn2$^{-/-}$ adipocytes (23), further suggesting a non-genomic regulation of RA signaling by Lcn2. Thus, we hypothesized that Lcn2 may localize to the plasma membrane and regulate RAR-α translocation to the plasma membrane. Differentiated brown and inguinal adipocytes were treated with insulin or insulin plus RA for 45 minutes, followed by isolation of the plasma membrane fraction. As expected, we found abundant Lcn2 in plasma membrane fractions isolated from brown and inguinal adipocytes from WT mice, and insulin treatment enhanced

plasma membrane abundance of Lcn2 (Fig. 6A). Interestingly, RAR-α translocation to the plasma membrane was also significantly increased in response to insulin stimulation in WT inguinal and brown adipocytes (Fig. 6A, 6B, 6D). Insulin-stimulated RAR-α translocation was not significant in $Lcn2^{-/-}$ inguinal adipocytes, suggesting Lcn2 mediates this effect (Fig. 6B, 6D). Surprisingly, treatment of adipocytes with insulin and RA together led to a reduction in plasma membrane RAR-α when compared to treatment with insulin alone (Fig. 6B). To understand if this RA-induced reduction in plasma membrane RAR-α is related to changes in overall RAR-α protein levels and also if Lcn2 deficiency affects overall levels of RAR-α protein expression, we looked at the effect of RA on the mRNA and protein expression levels of RAR-α in WT and $Lcn2^{-/-}$ inguinal adipocytes with or without rosiglitazone induction during differentiation. As shown in Fig 6C and 6E, mRNA and protein expression levels of RAR-α were not significantly different between WT and $Lcn2^{-/-}$ inguinal adipocytes under the basal and RA-treated conditions. RA treatment for 24 h resulted in a similar reduction in RAR-α protein levels in WT and $Lcn2^{-/-}$ inguinal adipocytes (Fig 6E). Together, these data suggest that RA-induced decrease in plasma membrane RAR-α likely results from decreased total RAR-α protein levels.

Discussion

Thermogenic adaptation in BAT in response to cold is correlated with decreased BMI and improved metabolic health (2-4). In response to thermogenic stimuli, WAT additionally recruits brown-like, “beige” adipocytes that are multilocular, have more mitochondria, and express higher levels of UCP1 (2,26). Our lab has previously found

that Lcn2 regulates thermogenesis in BAT via a non-adrenergic mechanism (18,21). In this study we sought to determine whether and how Lcn2 regulates beiging of WAT including beige adipocyte recruitment and activation.

Although β-adrenergic signaling facilitated by catecholamine binding to adrenergic receptors on the surface of adipocytes is the primary pathway regulating adipose tissue thermogenesis (2,6,7), alternative mechanisms independent of β-adrenergic stimulation have been reported. Insulin upregulates *Ucp1* gene expression through activation of P13K/Akt signaling in brown adipocytes and is known to have a permissive role in mediating diet-induced thermogenesis (33,34). Mice lacking insulin receptors in adipose tissue cannot maintain their body temperature (35), indicating insulin action is necessary for thermogenesis. Additionally, insulin has been shown to play a critical role in mitochondrial function by regulating mitochondrial protein synthesis, oxidative capacity and PGC-1α (36-38). However, the role of insulin in adipocyte thermogenesis, particularly with regard to the signaling pathway and mechanism that mediates insulin action, remains largely unexplored. Lcn2 expression is highly inducible in response to insulin (17), nonetheless the role of Lcn2 in thermogenesis under these conditions is not well elucidated. We found impairment in insulin-stimulated p38MAPK signaling and thermogenic gene expression in $Lcn2^{-/-}$ adipocytes. On the other hand, insulin treatment significantly increased p38MAPK phosphorylation and thermogenic gene expression in WT adipocytes. Moreover, we showed that recombinant Lcn2 can significantly upregulate thermogenic gene expression. This suggests that in response to insulin stimulation, Lcn2 is increased and may in turn mediate insulin-permissive action

on thermogenic signaling and gene expression. Impaired insulin-permissive action on signaling and gene expression may also contribute to the mitochondrial dysfunction seen in Lcn2$^{-/-}$ inguinal adipocytes, indicated by significantly reduced oxidative capacity and decreased NAD+/NADH ratio.

Chronic activation of PPARγ by rosiglitazone is known to recruit UCP1+ adipocytes, particularly in iWAT (5,8,9). Insulin is known to positively regulate PPARγ through increasing Akt-mTORC1 pathway activity and mTORC1 activation has been reported as necessary for beiging (28,39). Together this suggests that insulin pathway activation might regulate WAT thermogenesis in WT mice in part by promoting PPARγ activity. Our lab has previously demonstrated a decrease in insulin-stimulated Akt phosphorylation in Lcn2$^{-/-}$ adipocytes (18), suggesting Lcn2 promotes insulin pathway activation. However, it is not known whether Lcn2 is necessary for insulin signaling following rosiglitazone treatment. In this study, we showed that differentiation of adipocytes in response to the PPARγ agonist rosiglitazone was similar between WT and Lcn2$^{-/-}$ adipocytes. We further showed that chronic rosiglitazone treatment induced similar levels of *Ucp1* gene expression and UCP1 protein in WT and Lcn2$^{-/-}$ white adipocytes. This is consistent with our published studies showing that rosiglitazone treatment was able to rescue cold intolerance and UCP1 expression in BAT in Lcn2$^{-/-}$ mice (22). After rosiglitazone treatment during differentiation, phosphorylation of Akt and S6K in response to insulin stimulation was not different between WT and Lcn2$^{-/-}$ inguinal adipocytes. Interestingly, Lcn2$^{-/-}$ rosiglitazone-differentiated inguinal adipocytes remained unresponsive to acute treatment with insulin plus RA in regards to thermogenic

gene expression, indicating that PPARγ is not involved in the acute regulation of thermogenic signaling by Lcn2. Moreover, we did not observe induction of Akt and S6K phosphorylation in response to RA in both WT and Lcn2$^{-/-}$ rosiglitazone-differentiated inguinal adipocytes. This further supports insulin-stimulated Lcn2 secretion may regulate thermogenic signaling through mTORC1-independent mechanisms.

In addition to enhancing mitochondrial function to maximize thermogenic potential (36,37), insulin has been shown to augment the action of other thermogenic factors, including RA (40). RA synergizes with insulin to dramatically induce UCP1 expression in brown adipocytes, as well as sterol regulatory element-binding protein (SREBP) target gene *glucokinase* (*Gck*) in hepatocytes (40,41). However, the molecular mechanisms and signaling pathways that link the synergistic action of insulin and RA to thermogenesis remain largely unknown. We saw a synergistic effect on thermogenic gene expression when WT inguinal adipocytes were treated with a combination of insulin and RA, and this was intriguingly blunted in Lcn2$^{-/-}$ adipocytes. This suggests that Lcn2 is involved in the synergistic activity of insulin and RA, especially in regards to thermogenesis.

RA has been reported to increase thermogenesis independently of β-adrenergic signaling. RA directly binds to and assists the formation of RARα/RXR heterodimers, which translocate to the nucleus and increase transcription of UCP1 (11,12,14,16). Further studies have found RA may activate thermogenesis upstream of transcription by activating non-genomic RARα signaling pathways, in particular p38MAPK (15,16,30). In mouse embryonic fibroblasts, RA treatment has been shown to rapidly induce

translocation of RAR-α to plasma membrane lipid rafts, enabling RAR-α interaction with g-coupled proteins and resulting in increased p38MAPK activity (29). Taken together, these studies suggest that RA enhances UCP1 levels through both genomic and non-genomic mechanisms. Whether the effect of RA on white adipose tissue beiging is permissive, or is simply due to changes in RA action in response to thermogenic stimuli, remains unclear.

Lcn2 shares structural similarity with the lipocalin family of proteins, members of which have been shown to bind ligands including retinoids in a hydrophobic binding pocket common to the family (19,20). Previous studies in our lab have shown that Lcn2 regulates RA metabolism in adipose tissue but poorly binds RA in vitro (23), suggesting Lcn2 does not regulate RA activities by directly binding to it. Increased transcription of thermogenic genes in response to RA is known to be mediated by translocation of the nuclear transcription factor RAR-α from the cytosol to the nucleus (11,12,14,15). However, we have previously shown RA-stimulated translocation of RAR-α to the nucleus is normal in Lcn2-deficient adipocytes and not all RA-responsive genes are downregulated in the absence of Lcn2 (23), suggesting a more specialized mechanism by which Lcn2 regulates RA-induced thermogenic gene expression. In this study, we saw an increase in p38MAPK phosphorylation in response to a short, 30-minute treatment with RA in WT inguinal adipocytes, and this was significantly blunted in $Lcn2^{-/-}$ inguinal adipocytes. This indicates that Lcn2 is required for RA's ability to rapidly activate a non-genomic RARα-p38MAPK pathway. Lcn2 has been shown to bind to the plasma membrane of sperm and facilitate lipid raft rearrangement (31). Strikingly, we found both Lcn2 and RAR-α present on the plasma membrane in brown and inguinal adipocytes.

Insulin increases RAR-α levels on the plasma membrane in WT, but not Lcn2-deficient adipocytes, suggesting that Lcn2 is required for insulin-stimulated plasma membrane translocation of RAR-α. Interestingly, insulin treatment increased RAR-α levels to a greater extent than RA and insulin together in the plasma membrane of WT adipocytes. The reason for this is unknown, however, it may be related to degradation of RAR-α in response to RA-binding (42). Indeed, we also showed that RA treatment reduces total cellular RAR-α protein levels in WT and $Lcn2^{-/-}$ inguinal adipocytes (Fig 6E).

Based on our findings, we propose a novel model where in response to insulin, Lcn2 levels are increased and localize to lipid rafts on the plasma membrane (Fig. 7). Under insulin-stimulated conditions, the presence of Lcn2 on the plasma membrane promotes localization of RAR-α. This may be the mechanism by which RA facilitates phosphorylation of p38MAPK and thermogenic activation. Future studies are needed to determine whether Lcn2 binds intracellularly or extracellularly to the plasma membrane. It is also unknown whether Lcn2 directly interacts with RA or RAR-α at the plasma membrane or simply promotes translocation of RAR-α. Taken together, these results show that Lcn2 is a regulator of insulin's action on RA-induced thermogenic gene expression by promoting localization of RAR-α to the plasma membrane and thermogenic signaling through p38MAPK.

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Figure 1

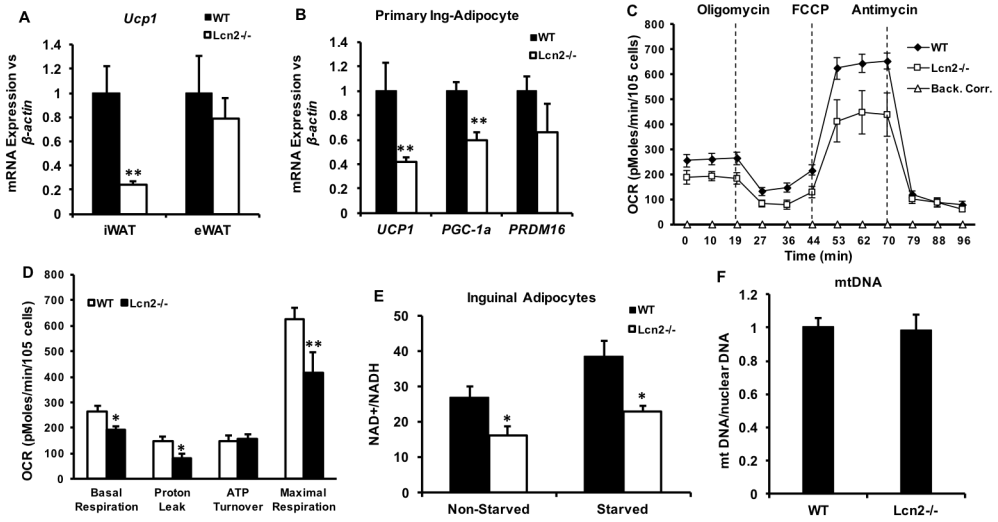

Figure 1. Thermogenic gene expression and mitochondrial function in Lcn2$^{-/-}$ WAT and inguinal adipocytes. (A) *Ucp1* gene expression in inguinal WAT (iWAT) and epididymal WAT (eWAT) from WT and Lcn2$^{-/-}$ mice on high-fat diet for 12 weeks, n=3-5/group. (B) Thermogenic gene expression in primary inguinal adipocytes isolated from WT and Lcn2$^{-/-}$ mice, n=3-5/group. (C and D) Oxygen consumption rate in differentiated inguinal adipocytes isolated from WT and Lcn2$^{-/-}$ mice, n=8/group. (E) NAD+/NADH in differentiated inguinal adipocytes isolated from WT and Lcn2$^{-/-}$ mice, n=3/group. (F) mtDNA relative to nuclear DNA in inguinal differentiated adipocytes from WT and Lcn2$^{-/-}$ mice, n=3/group. The cell culture experiments were repeated 2-3 times yielding similar results. The data are represented as mean +/- SEM. *P<0.05 vs WT; **P<0.01 vs WT.

Figure 2

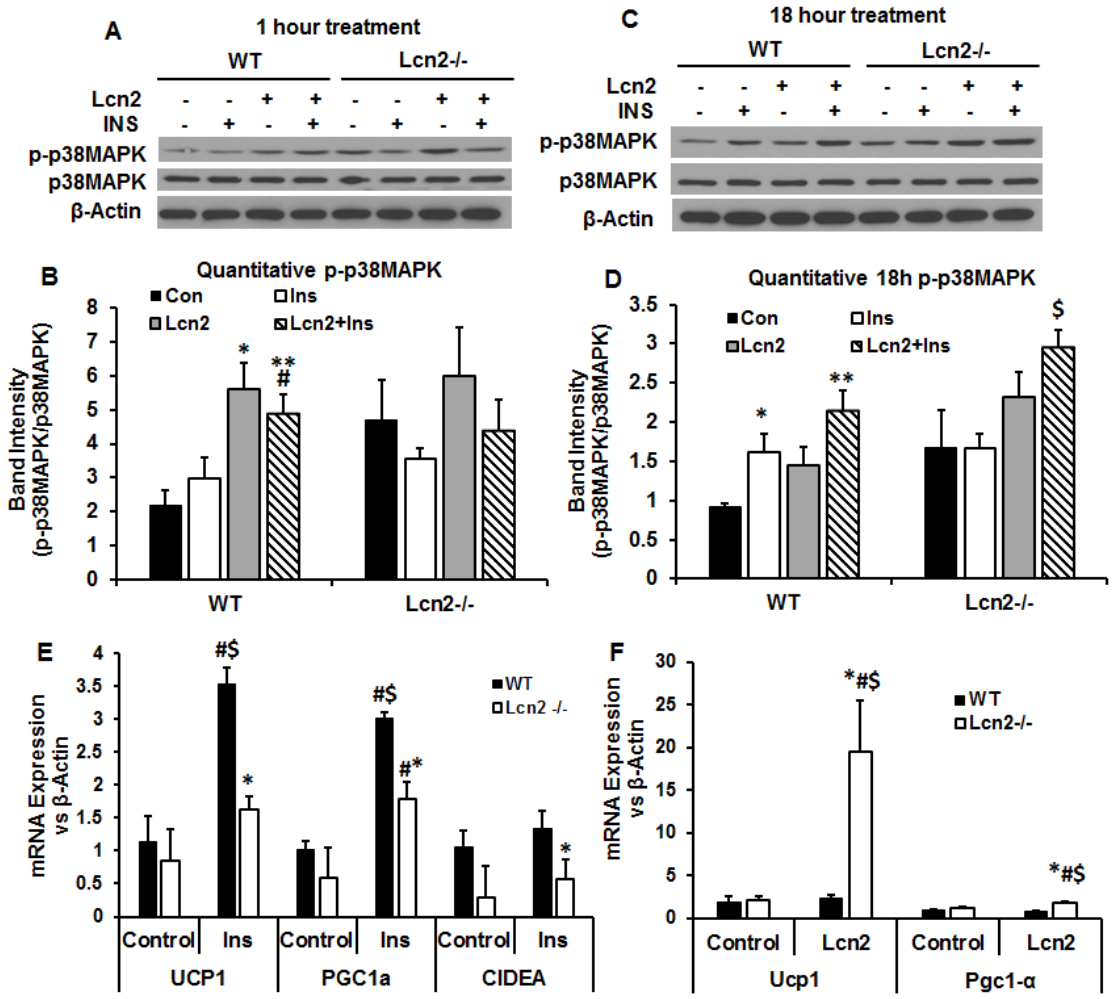

Figure 2. p38MAPK signaling and thermogenic gene expression in Lcn2$^{-/-}$ inguinal adipocytes under basal and insulin-stimulated conditions. (A and C) p38MAPK phosphorylation in differentiated inguinal adipocytes following one hour (A) and 18 hours (C) of treatment with insulin (INS) +/- 500 ng/mL recombinant Lcn2. (B and D) Quantification of band intensity of phosphorylated p38MAPK normalized to total p38MAPK for one hour treatment (B) and 18 hour treatment(D), n=3 independent experiments/group. (E) Gene expression of *Ucp1*, *Pgc-1α*, and *Cidea* following 18 hours treatment with or without insulin (INS), n=3/group. (F) Gene expression of *Ucp1* and *Pgc1-α* following 18 hours treatment with or without 500 ng/mL recombinant Lcn2, n=3/group. The cell culture experiments were repeated 2-3 times yielding similar results. The data are represented as mean +/- SEM. *P<0.05 vs WT of same group; **P<0.01 vs WT of same group; $P<0.05 vs WT Control; #P<0.05 vs Lcn2$^{-/-}$ Control.

Figure 3

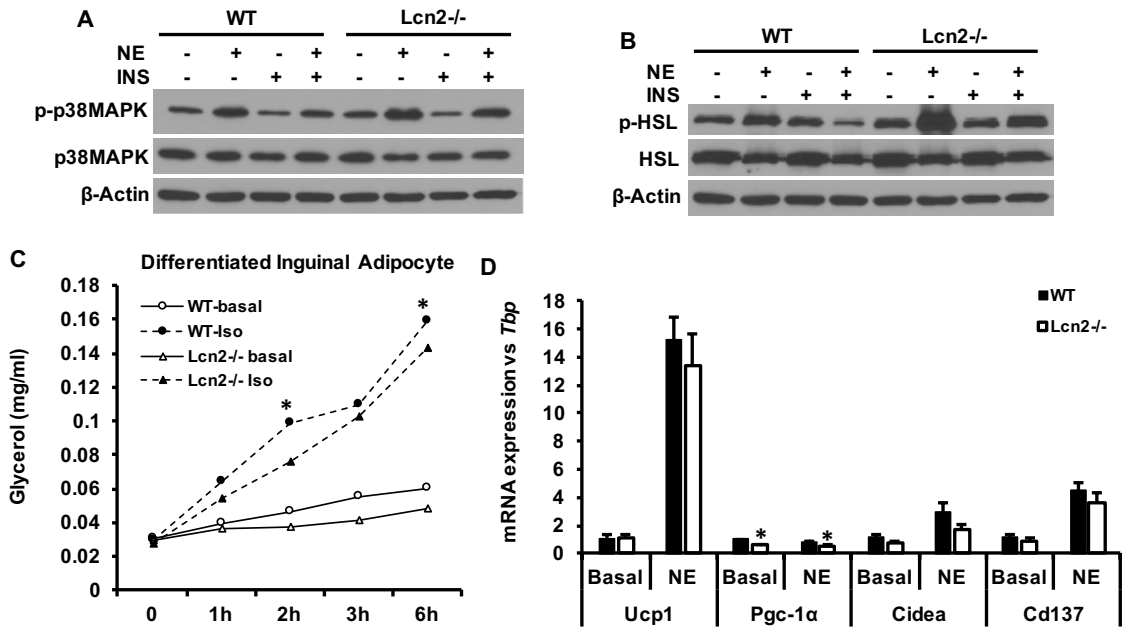

Figure 3. β-adrenergic signaling in Lcn2$^{-/-}$ inguinal adipocytes. (A and B) p38MAPK (A) and HSL (B) phosphorylation following treatment with 1 μM norepinephrine (NE) +/- insulin (Ins). (C) Glycerol release into cell supernatant from differentiated inguinal adipocytes treated with 1 μM isoproterenol (Iso). (D) Gene expression in differentiated inguinal adipocytes treated with 1 μM norepinephrine (NE) for 18 hours, n=3/group. Experiments were repeated 2 times yielding similar results. The data are represented as mean +/- SEM. *P<0.05 vs WT.

Figure 4

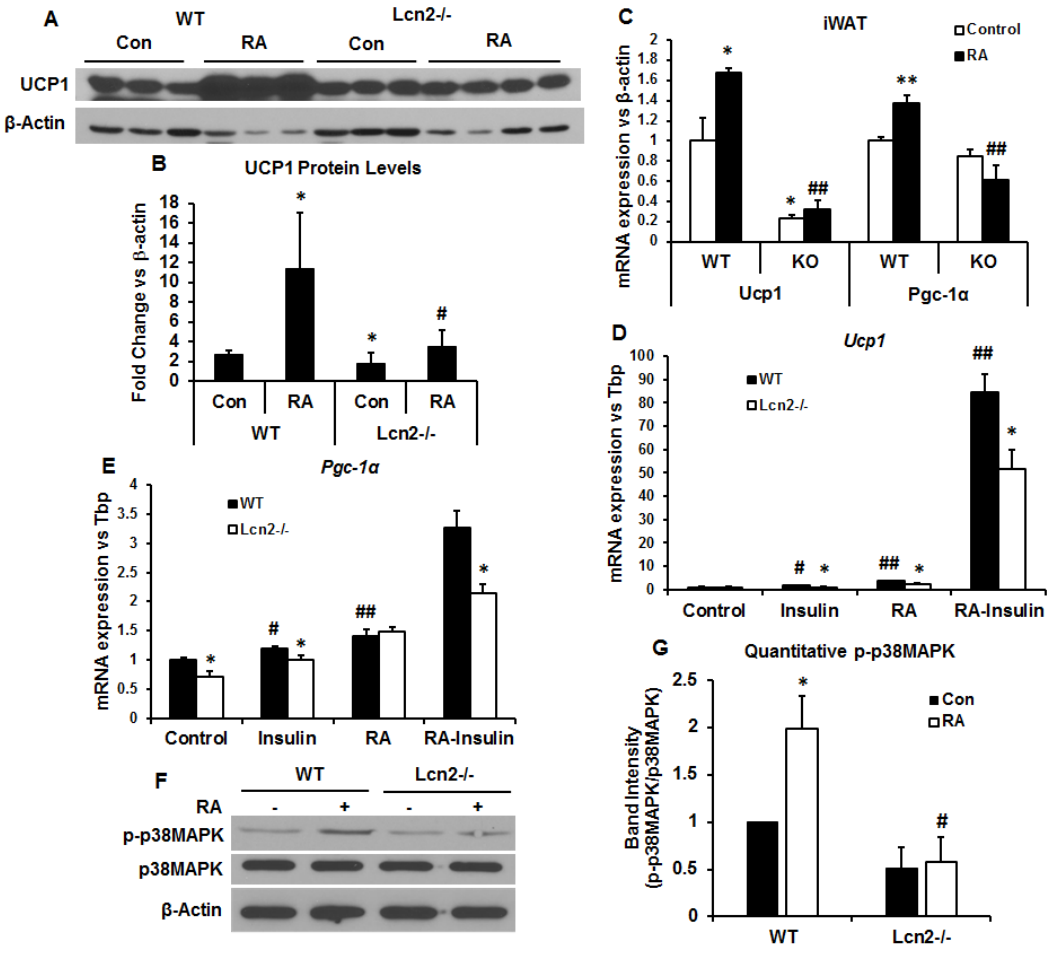

Figure 4. RA-induced thermogenesis in Lcn2$^{-/-}$ iWAT and adipocytes. (A-C) UCP1 protein levels (A and B) (n=3-4 mice/group) and *Ucp1* and *Pgc-1α* gene expression (C) (n=4-6 mice/group) in iWAT from WT and Lcn2-/- mice following oral gavage with RA for 14 days. *P<0.05 vs WT; #P<0.05 vs WT RA. (D and E) *Ucp1* (D) and *Pgc-1α* (E) gene expression in differentiated inguinal adipocytes following 24 hour treatment with insulin and 1 μM RA, n=3/group. *P<0.05 vs WT; #P<0.05 vs WT-Con. (F and G) p38MAPK phosphorylation in differentiated inguinal adipocytes following 30 minute treatment with 1 μM RA, n=4 independent experiments/group. For cell culture studies, experiments were repeated 2-3 times yielding similar results. The data are represented as mean +/- SEM. *P<0.05; **P<0.01; #P<0.05; ##P<0.01.

Figure 5

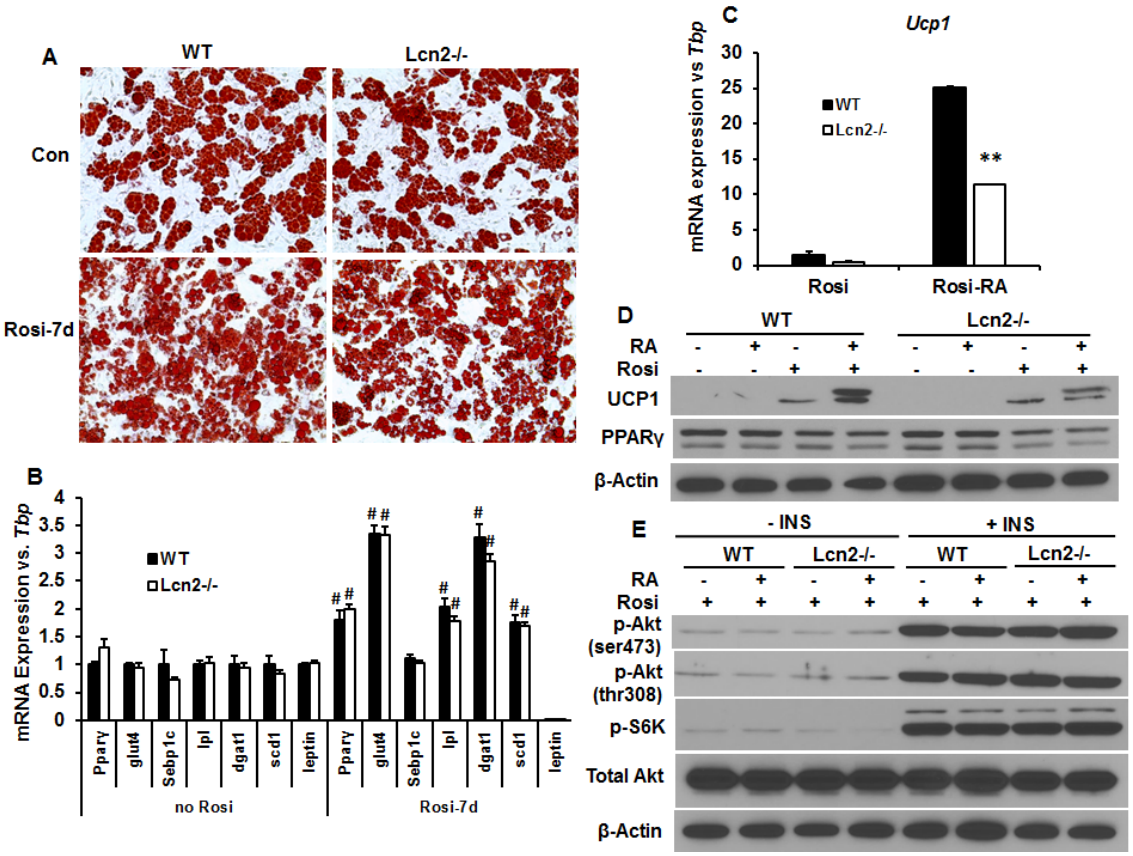

Figure 5: Effect of rosiglitazone on thermogenesis in Lcn2$^{-/-}$ inguinal adipocytes. (A) Oil red O Staining in differentiated inguinal adipocytes treated with or without 1 μM rosiglitazone (Rosi-7d) during the 7 days of differentiation to mature adipocytes. (B) Adipogenic gene expression in differentiated inguinal adipocytes treated with or without 1 μM rosiglitazone (Rosi-7d) during the 7 days of differentiation to mature adipocytes, n=3/group. (C and D) *Ucp1* gene expression (C) and protein levels (D) in differentiated inguinal adipocytes treated with 7 days of 1 μM rosiglitazone (Rosi) during differentiation followed by 24 hour treatment with 1 μM RA, n=3/group. (E) Levels of proteins involved in insulin signaling in differentiated inguinal adipocytes treated with 1 μM rosiglitazone (Rosi) during the 7 day differentiation to adipocytes followed by 1 μM RA or insulin for 30 minutes. Experiments were repeated 2-3 times yielding similar results. The data are represented as mean +/- SEM. **P<0.01 vs WT from similar treatment group; #P<0.05 vs no rosi treatment; ##P<0.01 vs no rosi treatment.

Figure 6

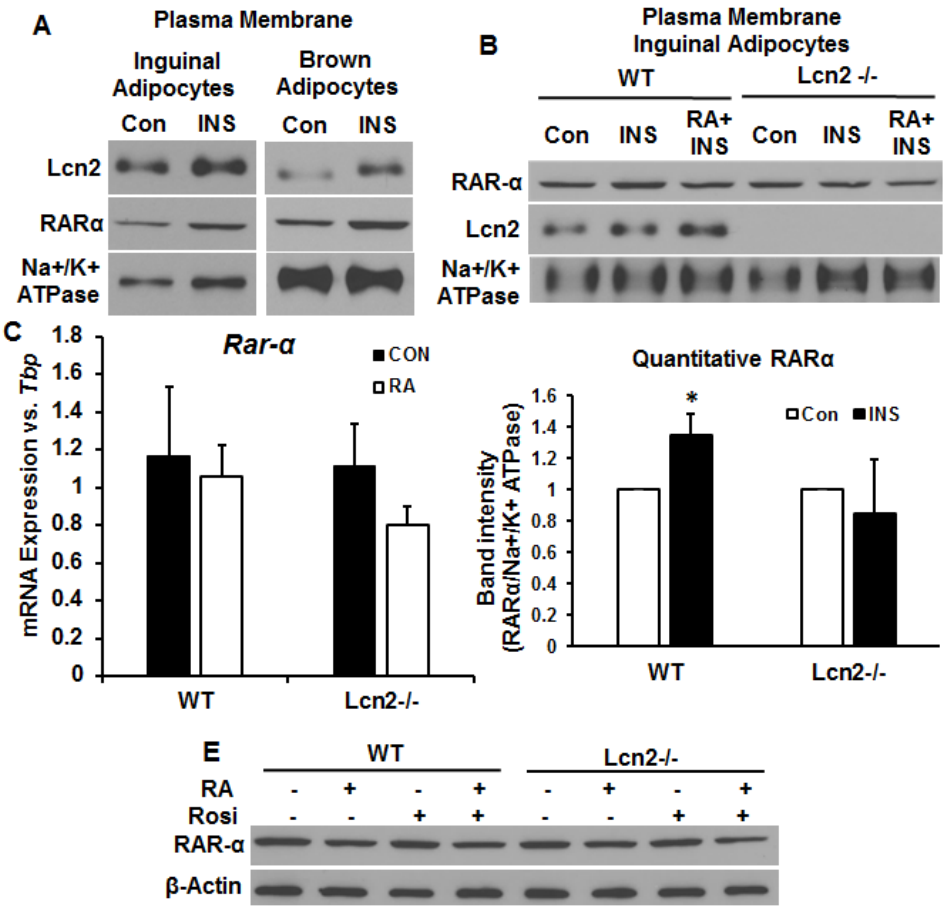

Figure 6. Plasma membrane localization of RAR-α in Lcn2$^{-/-}$ brown and inguinal adipocytes. (A) Levels of RAR-α and Lcn2 in plasma membrane isolated from WT brown adipocytes and WT inguinal adipocytes following 45 minute treatment with insulin. (B) Levels of RAR-α and Lcn2 in plasma membrane isolated from WT and Lcn2$^{-/-}$ inguinal adipocytes following 45 minute treatment with insulin +/- 1 μM RA. (C) *Rar-α* gene expression in differentiated inguinal adipocytes treated with 24 hours of 1 μM RA, n=3/group. (D) Quantification of basal and insulin-stimulated levels of RAR-α in plasma membrane isolated from WT and Lcn2$^{-/-}$ adipocytes following 45 minute treatment +/- insulin, n=4-5 independent experiments/group. (E) RAR-α protein levels in differentiated inguinal adipocytes treated with 1 μM rosiglitazone during the differentiation to adipocytes followed by 24 hour treatment with 1 μM RA. The data are represented as mean +/- SEM. *P<0.05 vs control.

Figure 7

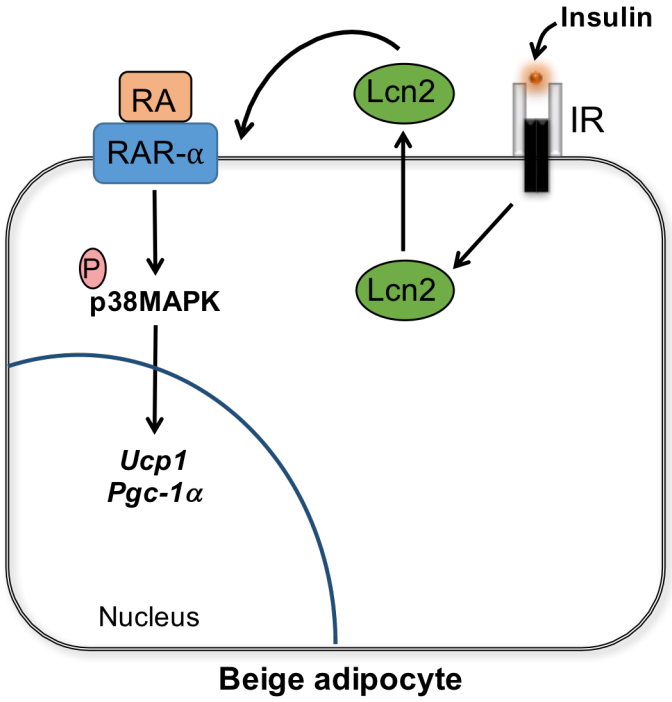

Figure 7: Proposed model for regulation of retinoic-acid induced thermogenic signaling by Lcn2. Lcn2 expression and secretion is induced by insulin. Lcn2 binds to lipid rafts in the plasma membrane and facilitates translocation of retinoic acid receptor-alpha (RAR-α) to the plasma membrane. Plasma membrane-bound RAR-α promotes phosphorylation of p38MAPK and thermogenic gene expression.

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Chapter 3

Overexpression of Lipocalin 2 in Adipose Tissue Improves Cold Tolerance and Beiging of White Adipose Tissue

Abstract

Mice deficient in lipocalin 2 (Lcn2) are more prone to diet-induced obesity, insulin resistance, and have impaired thermogenesis. However, it is unknown whether overexpression of Lcn2 in adipose tissue promotes thermogenesis and prevents obesity-associated metabolic disturbances. In this study, we generated ap2-promoter-driven Lcn2 transgenic (Tg) mice that overexpress Lcn2 in adipose tissue. When exposed to cold, Lcn2 Tg mice maintain a significantly higher body temperature when compared to WT mice. This is in addition to increased uncoupling protein 1 (*Ucp1*) and peroxisome proliferator-activated receptor-gamma coactivator-1α (*Pgc-1α*) gene expression in inguinal white adipose tissue (iWAT) in Lcn2 Tg mice, suggesting Lcn2 promotes iWAT beiging. Under non-stressed conditions, Lcn2 Tg mice have a trend towards a decreased respiratory exchange ratio (RER), decreased adiposity, and increased expression of genes involved in thermogenesis and fat oxidation in iWAT, suggesting they favor fat utilization. While total serum fatty acid levels are unchanged, Lcn2 Tg mice have a trend towards increased levels of the short-chain fatty acid (SCFA) propionate in serum. SCFAs function as signaling molecules and could mediate the metabolic changes in Lcn2 Tg mice, including suppression of lipogenesis and lipolysis in adipose tissue. In conclusion, overexpression of Lcn2 in adipose tissue regulates whole-body metabolism by increasing beiging in iWAT, leading to increased lipid oxidation and a decrease in adiposity.

Introduction

Obesity develops due to an imbalance between energy intake and energy expenditure. However, this rationale does not explain why it can be difficult to maintain a healthy body weight despite decreasing caloric intake and increasing physical activity (1,2). Several biological variables have been proposed as contributing to this discrepancy, including circulating factors secreted from adipose tissue (3). How these factors regulate energy expenditure and metabolism in response to environmental signals such as diet and metabolic stress is complex and requires further understanding.

In rodents and other hibernating mammals, non-shivering thermogenesis in brown adipose tissue (BAT) contributes to energy expenditure (4,5). In response to cold, expression of uncoupling protein 1 (UCP1) results in dissipation of the proton gradient across the inner mitochondrial membrane and conversion of chemical energy to heat. Increased fat oxidation to support this process leads to increases in whole body energy expenditure. During prolonged cold exposure, brown-like adipocytes develop in white adipose tissue (WAT) depots. These "beige" adipocytes have higher mitochondrial number, increased UCP1 expression, and are thermogenically competent (6,7). Beige adipose tissue activation also contributes to increased energy expenditure and oxidation of fatty acids and glucose during non-shivering thermogenesis (7). In addition to cold, several circulating factors have been identified as inducing thermogenesis in adipose tissue. For example, fibroblast growth factor 21 (FGF21) secreted from adipose tissue and cardiac natriuretic peptides both promote expression of UCP1 in WAT (8,9).

BAT has also been identified in the supraclavicular and neck region of humans, and presence of these depots is associated with a lower body mass index (BMI) (10,11).

Further, humans exposed to cold have increased blood flow and glucose uptake in BAT together with increased energy expenditure (12). Interestingly, cells from human BAT depots express a genetic profile more similar to rodent beige adipocytes as opposed to brown adipocytes (13). This suggests that increasing beiging of WAT is a potential therapeutic target for increasing energy expenditure and preventing obesity.

Lipocalin 2 (Lcn2) is a circulating factor secreted from adipose tissue in response to obesity, inflammation, and nutrient/growth signals (14). Our group has found that mice lacking Lcn2 gain more weight on a high-fat diet (HFD), develop more severe insulin resistance, and have impaired thermogenesis (15,16). *Law et al* found an opposite phenotype, wherein mice lacking Lcn2 are protected from developing insulin resistance in response to obesity (17). An additional study found only slight differences between wild-type (WT) and Lcn2 knock-out mice (18). More recently, bone-derived Lcn2 has been shown to improve insulin sensitivity and regulate food intake by suppressing appetite (19). This study supports a role for Lcn2 in the prevention of obesity and metabolic complications.

Previous studies utilized whole-body knock-out of Lcn2, so the exact contribution adipose tissue-derived Lcn2 makes to this phenotype is unknown. Further, there are no studies examining the role of Lcn2 overexpression, specifically in adipose tissue, on regulating whole-body metabolism, which is important for fully understanding the metabolic role of Lcn2. Therefore, the objective of this study is to determine whether Lcn2 overexpression in adipose tissue can have beneficial effects under non-stressed conditions and when mice are challenged with cold exposure and HFD feeding.

Materials and Methods

Generation of Adipocyte Fatty Acid-Binding Protein (aP2) Promoter-driven Lcn2 Transgenic Mice

A full length Lcn2 cDNA fragment was cloned to a pCI expression vector containing the 5.5 kb mouse aP2 promoter/enhancer by XhoI and Not I sites. The final plasmid is designated paP2–Lcn2. The integrity of the plasmid was confirmed by DNA sequencing of the ligation junctions.

The ap2-Lcn2 transgenic construct was digested with PvuI, after which the cloning vector and transgenic fragment were separated by agarose gel electrophoresis. The transgene was purified using a Qiagen plasmid purification kit (Qiagen) and then microinjected into the pronuclei of fertilized eggs collected from C57BL/6N mice (Charles River) at 2μg/ml in 10mM Tris-HCl (pH7.5), 0.1mM EDTA, and 100 mM NaCl. After culturing overnight in M16 culture medium (Millipore), those embryos reaching 2-cell stage of development were implanted into the oviducts of pseudopregnant foster mothers. Offspring born to the foster mothers were genotyped by PCR.

Animal Handling

Animals were housed at 22°C in a specific pathogen-free facility at the University of Minnesota. Animal studies were conducted with the approval of the University of Minnesota Animal Care and Use Committee and conformed to the National Institute of Health guidelines for laboratory animal care.

For the high-fat diet feeding study, WT and Lcn2 Tg mice from the same litter were housed at 22°C in 12:12-h light-dark cycle with free access to water. Male and female mice were fed either a regular chow diet (RCD) or a HFD (Bioserv, 60% of kilocalories from lard-derived fat) from 4-5 weeks of age. At 20-21 weeks of age, mice were sacrificed and tissues and serum were collected.

For the cold exposure study, WT and Lcn2 Tg male mice from the same litter were housed at 22C in a 12:12-h light-dark cycle with free access to water. Mice were fed a RCD from 6 weeks of age. For cold exposure, mice were fasted overnight prior to being placed in individual cages at 4°C with free access to water. Rectal temperature was recorded prior to placing in 4°C (Time 0), followed by rectal temperature measurement every 30 minutes for 4 hours. After 4 hours, mice were sacrificed and tissues and serum were collected.

Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

For GTT, mice were fasted overnight (18 hours) prior to the start of the test. For ITT, mice were fasted for 6 hours prior to the start of the test. Mice were given intraperitoneal glucose (0.75 g/kg) or insulin (0.75 units/kg) injections. Blood via the tail vein was tested for glucose level using an Ascensia Contour glucometer (Ascensia Diabetes Care, Parsippany, NJ) at 0-, 15-, 30-, 60-, 90-, and 120-minutes following glucose or insulin injection.

Metabolic Phenotyping

For the metabolic phenotyping study, male WT and Lcn2 Tg mice were fed a RCD from 6 weeks of age. At 16 weeks of age, mice were individually caged and food intake was measured over a 5-day period using a Biodaq food intake monitoring system (Research Diets Inc., New Brunswick, NJ). The final 2 days of food intake monitoring were averaged to determine total food intake in grams/day. Body composition was measured by EchoMRI (EchoMRI, Houston, TX) prior to indirect calorimetry measurement. Indirect calorimetry was measured over a 3-day period using the Oxymax Comprehensive Lab Monitoring System (Columbus Instruments, Columbus, OH). The final light and dark cycle were used to statistically determine differences in energy expenditure between genotypes. After measurement of indirect calorimetry, mice were sacrificed and tissues and serum were collected.

Histology

Inguinal white adipose tissues were fixed in 10% neutral buffered formalin (Thermo Scientific, Rockford, IL) for 24 hours and embedded in paraffin. After deparaffinization and rehydration, tissues were sectioned into 4 μm and stained with hematoxylin and eosin (H&E) using standard techniques. Images were taken under light microscopy.

Real-time PCR

Total RNA was isolated from tissue using TRIZOL reagent (Invitro, Carlsbad, CA). RNA was DNAase-treated prior to the synthesis of cDNA using Superscript II reverse transcription kit (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was

conducted using SYBR Green qPCR Master Mix (SABiosciences, Frederick, MD) with a StepOne Real-time PCR System (Applied Biosystem, Foster City, CA). The ΔΔCt method was used to calculate mRNA expression and *Tbp* or *Cyclophillin* served as an internal control.

Oil Red O Staining

Differentiated adipocytes were washed two times with phosphate buffered saline (PBS). Cells were fixed in Baker's Formalin for 30 minutes at 4°C. Following fixation, cells were stained with a 60% solution of Oil Red O (Sigma-Aldrich, Milwaukee, WI) in isopropyl alcohol for 10 minutes. Images were taken under light microscopy.

LC-MS Analysis of Fatty Acids

Serum sample or fatty acid standard was mixed with master reaction solution containing 1 mM DPDS, 1 mM TPP, and 1 mM HQ in ACN. The mixture was incubated at 60°C for 30 minutes and chilled on ice for 10 minutes. After terminating the reaction by mixing with H_2O, samples were centrifuged at 18,000xg for 10 minutes and the supernatant was collected. The derivatized sample was injected into an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) and separated by a BEH C18 column (Waters).

Serum Assays

Serum triglycerides were measured using the Stanbio LiquiColor® Triglycerides kit (Stanbio, Boerne, Texas) according to the manufacturer's instructions. Serum free fatty acids were measured using the Free Fatty Acids Half Micro Test (Roche, Mannheim, Germany) according to the manufacturer's instructions. Serum glycerol was measured using the Free Glycerol Kit (Sigma, Saint Louis, MO) according to manufacturer's instructions. Serum β-hydroxybutyrate was measured using the Stanbio LiquiColor® Beta-hydroxybutyrate kit (Stanbio, Boerne, Texas) according to the manufacturer's instructions.

Statistical Analysis

Values are reported as mean +/- standard error of the mean (SEM). Statistical significance was determined by two-tailed Student's *t* test, where a *P* value less than 0.05 was considered significant. For statistical analysis of the indirect calorimetry data, repeated-measure ANCOVA was used. The analysis included the absolute values with the associated body weight used as a covariate and the data presented as least-square means.

Results

1. Expression and distribution of Lcn2 in ap2-driven Lcn2 transgenic mice.

To generate aP2-promoter-driven Lcn2 transgenic mice, linearized aP2-Lcn2 DNA fragment was microinjected into the pronuclei of fertilized eggs collected from

C57BL/6N mice. Two transgenic founder mice were identified by PCR genotyping (Fig. 1A). We determined Lcn2 transgene expression at the protein and gene level.

When compared to WT mice, protein levels of Lcn2 were increased in adipose tissue depots from Lcn2 transgenic (Tg) mice following overnight fasting (Fig. 1B). Interestingly, there was a more substantial increase in Lcn2 in BAT from Tg mice when compared to the iWAT and eWAT. There was no difference in Lcn2 protein levels in liver, kidney, lung, spleen or brain when comparing Tg mice to WT (Fig. 1B, 1D). This indicates the ap2-driven Lcn2 transgene successfully overexpresses Lcn2 exclusively in adipose tissue depots.

Our group has previously found differential expression of Lcn2 in adipose tissue depots from WT male and female mice (20). We therefore examined abundance of Lcn2 in several tissues from WT and Lcn2 Tg male and female mice to determine whether Lcn2 expression in Tg mice was dependent on sex. There was no difference between genotype or sex when comparing Lcn2 in liver, brain and lung (Fig. 1E). Levels of Lcn2 overexpression in BAT and eWAT appear to be similar when comparing male and female Lcn2 transgenic mice (Fig. 1C). However, levels of Lcn2 are markedly higher in iWAT from female mice versus male mice (Fig. 1C), which may explain why there is no difference in iWAT Lcn2 levels in WT and Tg female mice. Analysis of serum showed Lcn2 levels in the circulation were not significantly different between WT and Tg mice (Fig. 1F). This suggests that the levels of overexpressed Lcn2 in adipose tissue are not high enough to change circulating Lcn2 levels.

2. Lcn2 Tg mice have improved adaptation to cold and beiging of iWAT.

We have previously found that mice deficient in Lcn2 are unable to maintain their body temperature at 4°C and have impaired thermogenesis (16). Therefore, we sought to determine whether overexpressing Lcn2 in adipose tissue can promote thermogenesis and improve cold adaptation. We placed RCD-fed WT and Lcn2 Tg mice at 4°C and measured body temperature every 30 minutes for 4 hours. Both room temperature and cold-exposed mice were fasted overnight prior to and during the cold exposure test. After 4 hours of exposure to 4°C, Lcn2 levels in BAT and iWAT are increased in WT mice (Fig. 2A). When compared to WT mice, Tg mice express higher levels of Lcn2 protein in BAT and iWAT at room temperature and in response to cold (Fig. 2A). Lcn2 gene expression in BAT is significantly increased under both room temperature and cold conditions in Tg mice (Fig. 2B). In iWAT, Tg mice have increased Lcn2 gene expression versus WT at room temperature, but not under cold conditions (Fig. 2C). At 150 minutes and 180 minutes, Tg mice maintained a significantly higher body temperature when compared to WT mice (Fig. 2D). This suggests that mice overexpressing Lcn2 in adipose tissue in response to cold produce more heat, potentially through increased thermogenesis in adipose tissue.

Interestingly, thermogenic genes were not increased in BAT from Tg mice, but *Ucp1* and peroxisome proliferator-activated receptor-gamma coactivator-1α (*Pgc-1α*) gene expression was significantly higher in Lcn2 Tg iWAT (Fig. 2E and 2F). This could point towards beiging of iWAT as a mechanism to support increased body temperature in response to cold in Lcn2 Tg mice.

3. Lcn2 Tg mice have altered whole-body metabolism under non-stressed conditions.

Interestingly, *Ucp1* expression was also increased in Lcn2 Tg iWAT under room temperature conditions (Fig. 2F), suggesting Lcn2 has a beiging effect. As UCP1 activity contributes to energy expenditure (7), we next investigated whether overexpression of Lcn2 in adipose tissue influences energy expenditure under room temperature conditions. RCD-fed, 16-week-old, WT and Lcn2 Tg mice were placed in metabolic chambers for three days to analyze energy expenditure via indirect calorimetry. Prior to indirect calorimetry, we analyzed body composition of the mice by EchoMRI. Body composition analysis revealed no difference in fat, lean, or total mass in Lcn2 Tg mice relative to WT mice (Fig. 3A).

Interestingly, while whole body oxygen consumption (VO_2) was unchanged in Lcn2 Tg mice, carbon dioxide output (VCO_2) was significantly decreased during the dark phase (Fig. 3B and 3C). A similar decrease in VCO_2 output compared to VO_2 consumption was previously reported in mice fed a ketogenic diet (21), pointing towards potential alterations in energy utilization preference. In line with decreased VCO_2 production, a trend towards a decrease in respiratory exchange ratio (RER) was also seen in Lcn2 Tg mice during the dark phase, indicating a possible increase in fat utilization when compared to WT mice (Fig. 3D). There was no difference in total food intake or activity level between WT and Tg mice (Fig. 3E and 3G). Although WT and Tg mice had no difference in total food intake, bouts/day were significantly decreased in Tg mice, indicating Tg mice eat less frequently than WT mice (Fig. 3F). This could potentially

signify a difference in satiety or behaviors surrounding food intake in response to overexpression of Lcn2 in adipose tissue.

Overexpression of UCP1 in adipose tissue has previously been reported to prevent obesity (22). As Lcn2 Tg mice have higher *Ucp1* expression in iWAT (Fig. 2D), we hypothesized Tg mice may be protected from weight gain on a HFD. However, when female and male WT and Lcn2 Tg mice were challenged to HFD feeding for 16 weeks, there was no difference in body weight, adipose tissue weight, or insulin sensitivity and glucose tolerance (Supplementary Fig. 1 and 2). While serum insulin, glycerol and triglycerides were increased in response to HFD, there was no difference between genotypes (Supplementary Figure 3A-3C). HFD downregulated *Gsta4* expression in WT mice and this was slightly, but significantly, attenuated in Tg mice (Supplementary Fig. 3E). Together, this data indicates overexpressing Lcn2 in adipose tissue has minimal impact on the effects of HFD in young animals.

4. *Lcn2 Tg mice have reduced adiposity and increased mitochondrial metabolism in adipose tissue under non-stressed conditions.*

The above data suggests that overexpression of Lcn2 in adipose tissue can stimulate beiging of iWAT and potentially fat oxidation in the absence of beiging agents such as cold. Next, we examined whether Lcn2 overexpression can change adipose tissue plasticity and oxidative metabolism of adipose tissue under non-stressed conditions. We found Lcn2 Tg mice had a trend towards a decrease in body weight at sacrifice (Fig 4A). Tissue weight of all three fat depots (BAT, iWAT, and eWAT) were significantly decreased in Lcn2 Tg mice when compared to WT mice (Fig. 4B). As the EchoMRI

results showed no change in total body fat, this could indicate redistribution of fat in non-adipose tissues. However, we saw no difference in tissue weight in liver, muscle, or heart (Fig. 4B). As body composition by EchoMRI was performed prior to placing mice in metabolic cages for 3 days, it is possible that the unfamiliar environment of the metabolic cages led to changes in body weight and body fat distribution that are reflected in the body and tissue weight at sacrifice.

We next looked at histology of adipose tissue to determine differences in cellular plasticity. Adipose tissue H&E staining showed a larger number of smaller adipocytes in Tg mice iWAT when compared to WT (Fig. 4C). *In vitro* differentiation of iWAT-derived stromal vascular cells to adipocytes showed no difference in adipogenic capability and lipid accumulation as measured by oil red o staining (Fig. 4D). This indicates changes in adipocyte size and number in Tg mice are not due to altered adipogenesis, but rather may be due to changes in thermogenesis or lipid metabolism in iWAT.

To determine whether Lcn2 overexpression is sufficient to drive beiging and fatty acid oxidation under non-stressed conditions, we next looked at the mRNA expression of genes involved in thermogenesis and oxidative metabolism in BAT and iWAT. Lcn2 Tg mice had an increase in expression of the thermogenic gene *Ucp1* and genes involved in fatty acid oxidation including peroxisome proliferator-activated receptor alpha (*Pparα*) and cytochrome c oxidase subunit 8b (*Cox8b*) (Fig. 4E). In BAT from Tg mice, while *Ucp1* gene expression was unchanged (Fig. 4F), expression of carnitine palmitoyltransferase 1 (*Cpt1*) and *Cox8b* genes were significantly increased under room temperature conditions. These results suggest that overexpression of Lcn2 in adipose

tissue promotes beiging and stimulates fat oxidation in iWAT without additional beiging agents, suggesting Lcn2 could function as a beiging factor.

5. Lcn2 Tg mice have an altered serum fatty acid profile.

Decreased VCO_2 output versus VO_2 consumption in Tg mice mirrors a similar metabolic profile found in mice fed a ketogenic diet (21). Additionally, a trend towards a decrease in RER indicates increased fat utilization. To better understand metabolic status in Lcn2 Tg mice, we profiled fatty acids and ketone bodies in serum from RCD-fed mice under the fed state using mass spectrometry. There was no change in serum levels of β-hydroxybutyrate in Lcn2 Tg versus WT mice (Fig. 5A). Interestingly, there was a trend towards an increase in serum levels of the short-chain fatty acid (SCFA) propionate in Lcn2 Tg mice, but no significant change in acetate or butyrate was observed (Fig. 5B-5D). There was a trend towards a decrease in the medium-chain fatty acids caproic acid (C6:0) and caprylic acid (C8:0) (Fig. 5E). This was accompanied by a significant increase in linoleic acid (C18:2), and the odd chain unsaturated fatty acid C17:1 (Fig. 5F, 5G). Despite changes in fatty acid composition, total serum fatty acid levels were similar between WT and Tg mice (Supplementary Fig. 4B). This data suggests that changes in fatty acid composition, particularly SCFAs, may play a role in regulating the metabolic effect seen in Lcn2 Tg mice.

We next examined serum levels of ketone bodies and lipids in Lcn2 Tg mice following cold adaptation. Serum levels free fatty acids were significantly increased after 4 hours of acute cold exposure in WT mice, but not Tg mice (Supplementary Fig. 4D). Serum triglyceride levels were not increased following cold exposure in WT or Tg mice

(Supplementary Fig. 4E). As Tg mice have increased oxidative and *Ucp1* gene expression in iWAT (Fig. 2F and 4E), the lack of increase in serum lipids following cold-exposure could indicate increased utilization of lipids for oxidation in adipose tissue.

6. Lcn2 Tg mice display metabolic changes characteristic of SCFA signaling.

Short-chain fatty acids (SCFAs) are produced by resident microbes in the gut and can act both as energy substrates and signaling molecules to regulate metabolism (23). SCFAs act through free fatty acid receptors 2 and 3, which are g-protein coupled receptors that are highly expressed in adipose tissue (23,24). SCFAs have been shown to increase expression of UCP1 and PGC-1α, increase fatty acid oxidation, and inhibit both lipolysis and lipogenesis (23-25). As we have shown above, serum levels of the SCFA propionate are increased in Tg mice (Fig. 5B). Therefore, we investigated whether metabolic changes associated with elevated SCFA levels are present in Tg mice. It has been reported that SCFAs inhibit lipolysis and lipogenesis (23,24), leading us to examine lipolytic and lipogenic genes in BAT and iWAT. Interestingly, Lcn2 Tg mice had significantly decreased expression of hormone sensitive lipase (*Hsl*) and fatty acid synthase (*Fasn*) in BAT (Fig. 6A) and *Hsl*, adipose triglyceride lipase (*Atgl*) and citrate synthase (*Cs*) in iWAT (Fig. 6B) when compared to WT mice under non-stressed conditions. Moreover, the cold-stimulated increases in HSL phosphorylation in iWAT (Fig. 6C and 6D) and serum glycerol levels (Fig. 6E) seen in WT mice were diminished in Lcn2 Tg mice. Together, these changes are consistent with the reported effects of SCFAs, suggesting that the metabolic phenotype of Lcn2 Tg mice may be related to changes in SCFAs.

Discussion

Increased amount and activity of BAT is associated with energy expenditure and a decrease in BMI (5,11,12). Our group has previously found that mice deficient in Lcn2 are cold intolerant and have impaired thermogenic programming (15,16). Lcn2 knock-out mice are also more susceptible to insulin resistance and weight gain during HFD feeding (15). This suggests that Lcn2 may promote thermogenesis in response to cold and protect against diet-induced obesity. In this study, we developed an ap2-promoter-driven Lcn2 transgenic mouse model to determine whether overexpression of Lcn2 in adipose tissue has beneficial effects on energy metabolism in response to cold exposure and high-fat diet.

We found Lcn2 Tg mice had an ability to maintain significantly higher body temperature than WT mice during acute cold exposure (4°C). As *Ucp1* gene expression in iWAT is significantly higher in Lcn2 Tg mice at room temperature, it is possible that Lcn2 Tg mice can more rapidly activate thermogenesis in iWAT in response to cold. After cold exposure, *Pgc-1α* gene expression was significantly higher in iWAT in Lcn2 Tg mice versus WT. During cold, PGC-1α activity promotes mitochondrial biogenesis and coactivates peroxisome proliferator-activated receptor gamma (PPARγ) to induce *Ucp1* transcription (26). This suggests that, in Lcn2 Tg mice, PGC-1α may be mediating some or all of the effect on cold adaptation and *Ucp1* expression in iWAT.

Interestingly, we saw no difference in BAT *Ucp1* gene expression between WT and Lcn2 Tg mice at both room temperature and after cold exposure. It has previously been reported that UCP1 is already highly abundant at room temperature in murine BAT

due to thermal stress, as thermoneutrality in mice is 30°C (27). This leaves the possibility that Lcn2 Tg mice may express higher levels of UCP1 in BAT at thermoneutrality. Regardless, higher body temperature and increased thermogenic gene expression in iWAT during cold exposure indicates improved cold adaptation in Tg mice versus WT.

Overexpression of UCP1 in adipose tissue has been shown to protect against obesity (22). Despite Lcn2 Tg mice having increased expression of *Ucp1* in iWAT, we found no differences in body weight, adiposity or insulin sensitivity between WT and Lcn2 Tg mice following HFD-feeding. As HFD has previously been found to significantly increase Lcn2 levels in WT mice (15), it is possible that Lcn2 levels become similar between WT and Lcn2 Tg mice in response to HFD and the effect of overexpressing Lcn2 is diminished under this condition.

While total fat mass as measured by EchoMRI was unchanged in Lcn2 Tg mice, adipose tissue weight was significantly decreased. Adipocyte size in iWAT from Lcn2 Tg mice is also smaller, with no change in differentiation rate, suggesting the smaller size is not due to defective adipogenesis. A trend towards decreased RER suggests that Lcn2 Tg mice are oxidizing more fat, which could explain the decrease in adiposity. Indeed, we saw an increase in genes involved in fat oxidation, including *Ppar*α, in iWAT from Lcn2 Tg mice. These results are consistent with our previous research showing Lcn2 knock-out mice have impaired fat oxidation and increased adiposity (15,16).

From measurement of whole body energy expenditure in WT and Lcn2 Tg mice, we found Lcn2 Tg mice have decreased CO_2 output, with no change in O_2 consumption during the dark, or active, cycle. A similar phenotype was seen during both the light and dark cycle in rats fed a ketogenic diet (21), leading us to examine ketone body levels in

Lcn2 Tg mice. Ketone esters have also been shown to increase *Ucp1* expression and activity in adipose tissue, which could explain the increase in iWAT *Ucp1* gene expression in room temperature Tg mice (28). However, serum levels of β-hydroxybutyrate in Lcn2 Tg mice were largely unchanged under both room temperature and cold conditions.

Interestingly, serum levels of the SCFA propionate trended towards an increase in Lcn2 Tg mice. The odd-chain fatty acid C17:1, which can be synthesized from or catabolized to propionate and is associated with a decreased incidence of type II diabetes (29), is also significantly increased in Lcn2 Tg mice. SCFAs have been reported to increase *Ucp1* and *Pgc-1α* gene expression and fatty acid oxidation, consistent with the changes we saw in iWAT from Lcn2 Tg mice (23,25,30). Further investigation found decreased lipolysis in Lcn2 Tg mice, which is also consistent with reported metabolic effects of SCFAs (24,30). This suggests that SCFAs, specifically propionate, may be mediating some of the metabolic changes in Lcn2 Tg mice. Recent studies have found that Lcn2 secreted from intestinal epithelial cells regulates the gut microbiome (31,32), which could alter SCFA production. Based on our results in Lcn2 Tg mice, this raises the question of whether adipose tissue-derived Lcn2 can regulate the gut microbiome as well to mediate the effects seen on metabolism and beiging of iWAT. It is also possible that Lcn2 may act as an effector of SCFAs, directly mediating metabolic function of SCFAs in thermogenic adipose tissue. However, this hypothesis requires further investigation.

In summary, we found that overexpression of Lcn2 in adipose tissue led to improved cold adaptation with increased *Ucp1* gene expression in iWAT, altered whole-body energy expenditure with a trend towards fat oxidation, increased oxidative gene

expression, decreased adipose tissue weight and adipocyte size, and modified serum fatty acid composition. Together, this data suggests that Lcn2 may increase beiging in iWAT, leading to increased lipid oxidation and a decrease in adiposity. More research needs to be done to determine whether increased *Ucp1* gene expression in Lcn2 Tg iWAT leads to a functional increase in oxidation in this tissue. It is also unclear whether BAT thermogenesis or Ucp1-independent thermogenic mechanisms contribute to the elevated body temperature seen in cold-exposed Lcn2 Tg mice. Lastly, whether and how Lcn2 secreted from adipose tissue can regulate SCFA production by gut microbiota to mediate the effects seen on metabolism in Lcn2 Tg mice requires further investigation.

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Figure 1

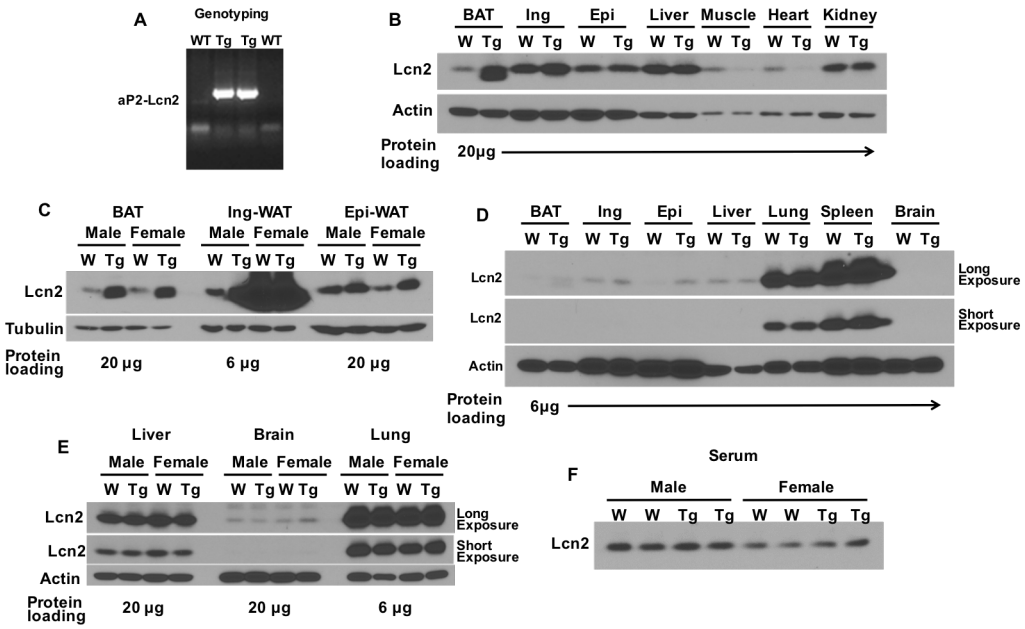


Figure 1: Expression and distribution of Lcn2 in ap2-promoter-driven Lcn2 transgenic mice. (A) Genotyping Lcn2 transgenic mice. (B and D) Tissue distribution of Lcn2 protein in male WT (W) and Lcn2 Tg (Tg) mice. (C and E) Comparison of tissue distribution of Lcn2 protein in male and female WT (W) and Lcn2 Tg (Tg) mice. (F) Protein levels of Lcn2 in serum from male and female WT (W) and Lcn2 Tg (Tg) mice.

Figure 2

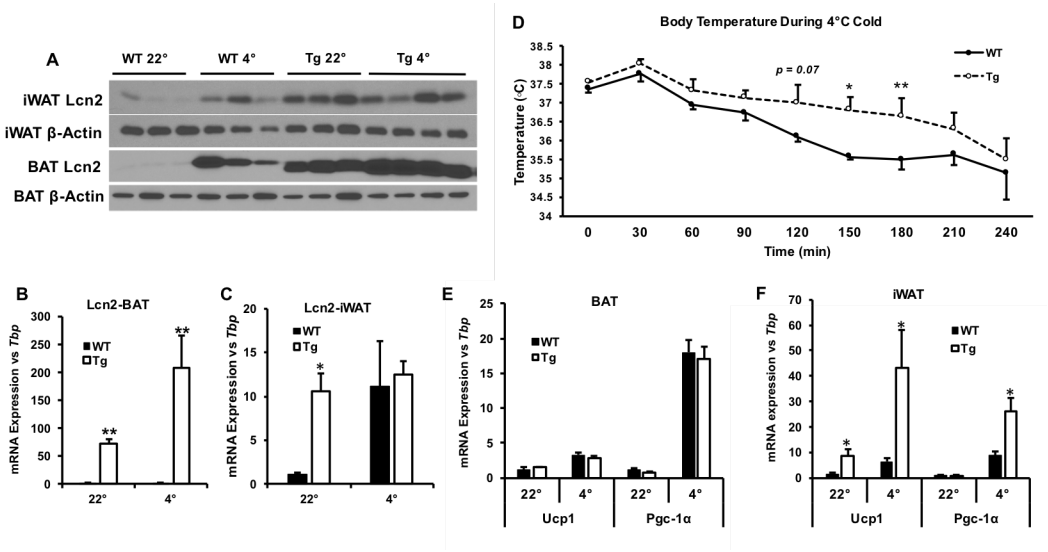


Figure 2: Cold adaptation in Lcn2 transgenic mice. (A) Protein levels of Lcn2 in wild-type (WT) and Lcn2 transgenic (Tg) mice at room temperature (22°C) and following four hours of cold exposure (4°C). (B and C) Lcn2 gene expression in (B) brown adipose tissue (BAT) and (C) inguinal white adipose tissue (iWAT) at 22°C and after four hours of cold exposure (4°C), n=3 mice/group. (D) Body temperature of WT and Lcn2 Tg mice measured by rectal thermometer every 30 minutes during four hours of cold exposure, n=3-5 mice/group. (E and F) Gene expression of *Ucp1* and *Pgc-1α* at room temperature and following four hours of cold exposure in (E) BAT and (F) iWAT from WT and Lcn2 Tg mice, n=3 mice/group. *p<0.05 vs WT. **p<0.01 vs WT.

Figure 3

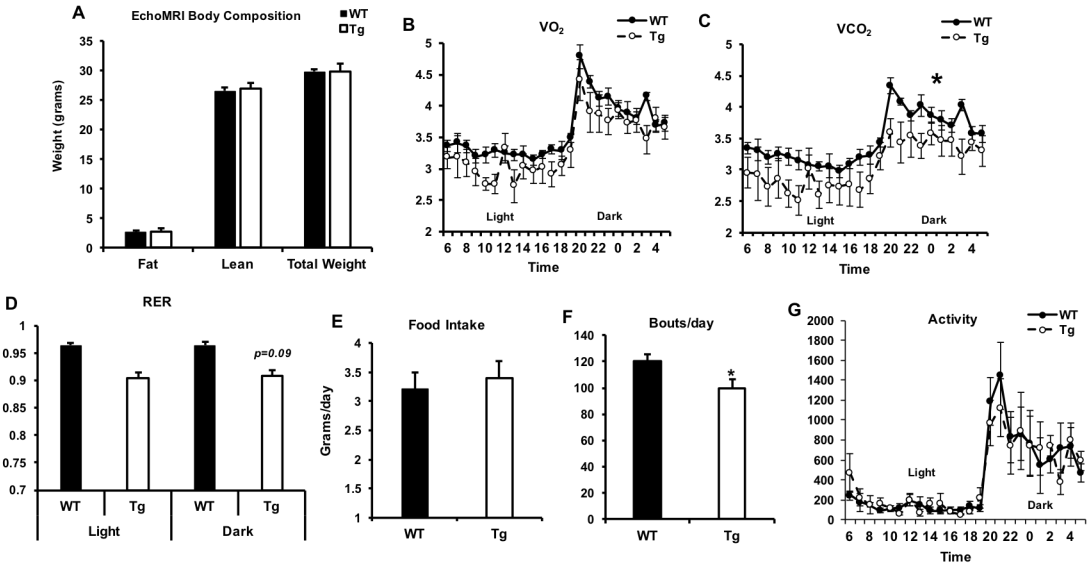


Figure 3: Energy expenditure in Lcn2 transgenic mice. (A) Body composition of WT and Lcn2 Tg (Tg) mice as measured by EchoMRI prior to indirect calorimetry, n=5-7 mice/group. (B and C) Oxygen consumption (VO_2) and carbon dioxide output (VCO_2) from WT and Lcn2 Tg mice, n=5-7 mice/group. Values represent one day of measurement during light cycle (6-20h) and dark cycle (20-5h). (D) Respiratory exchange ratio (RER) during light phase and dark phase, n=5-7 mice/group. (E) Total food intake averaged over 2 days, n=5-7 mice/group. (F) Food bouts per day, averaged over 2 days, n=5-7 mice/group. (G) Activity in WT and Lcn2 Tg mice, n=5-7 mice/group. Values represent one day of measurement. *$p<0.05$ vs WT.

Figure 4

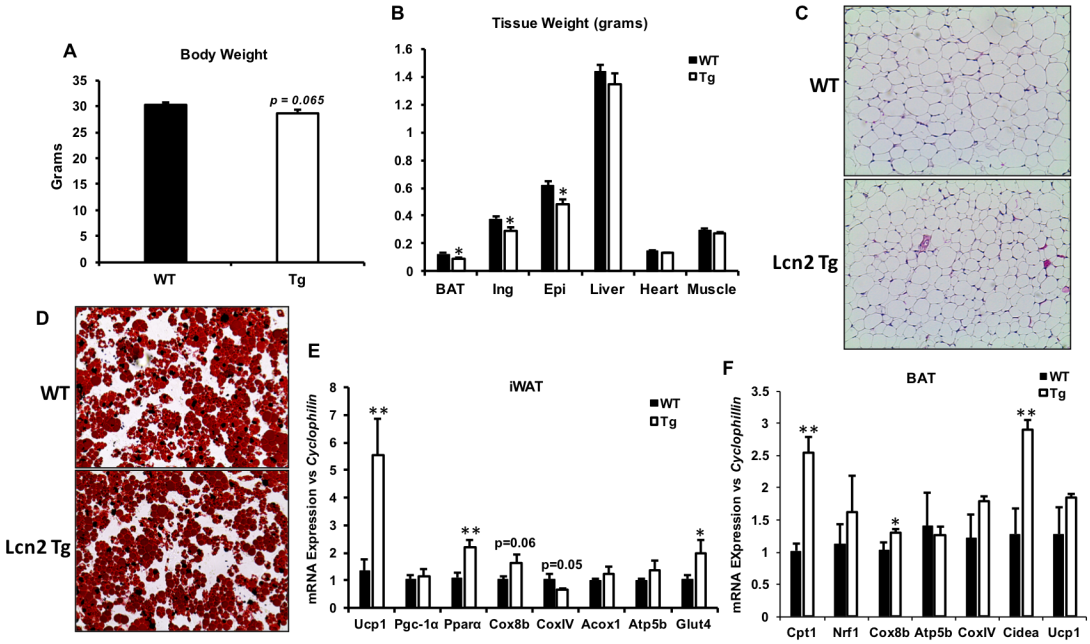


Figure 4: Adiposity and oxidative gene expression in Lcn2 transgenic mice. (A) Body weight of WT and Lcn2 Tg (Tg) mice following indirect calorimetry, n=5-7 mice/group. (B) Tissue weight of WT and Lcn2 Tg (Tg) mice following indirect calorimetry, n=5-7 mice/group. (C) H&E staining of inguinal white adipose tissue (iWAT) sections from WT and Lcn2 Tg mice, 20X magnification. (D) Oil red o staining of differentiated adipocytes isolated from iWAT of WT and Lcn2 Tg mice. (E and F) Oxidative gene expression in (E) iWAT and (F) BAT from WT and Lcn2 Tg (Tg) mice, n=3-4 mice/group. *p<0.05 vs WT. **p<0.01 vs WT.

Figure 5

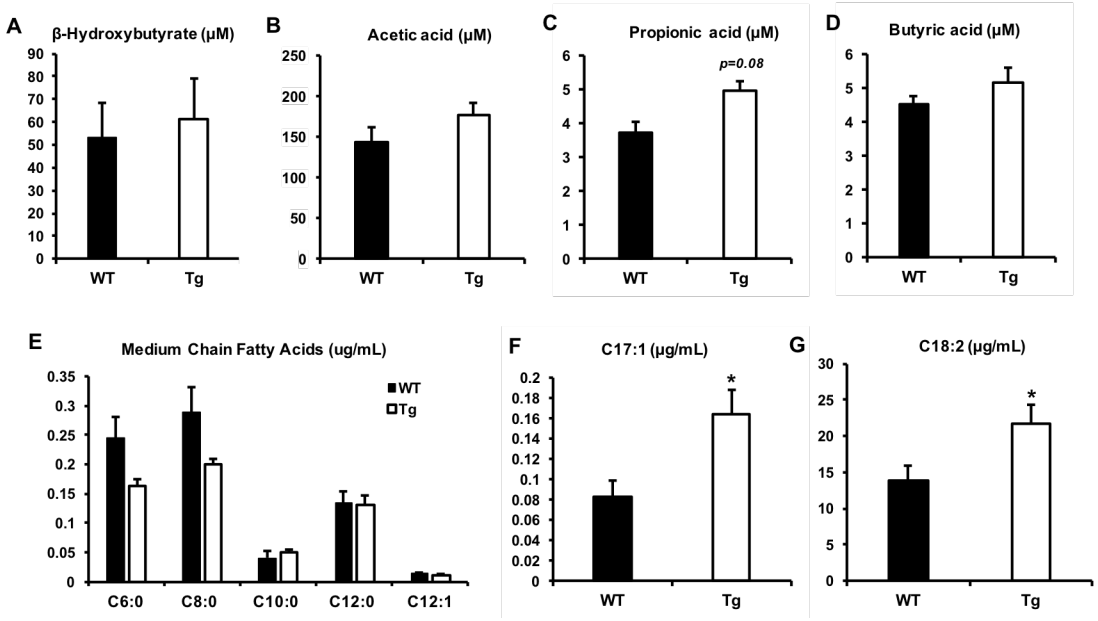


Figure 5: Serum lipid profile in Lcn2 transgenic mice. (A-E) Serum levels of (A) the ketone body, β-hydroxybutyrate, (B) the short-chain fatty acid (SCFA), acetic acid, (C) the SCFA, propionic acid, (D) the SCFA, butyric acid, (E) medium-chain fatty acids, (F) the long-chain fatty acids C17:1, and (G) C18:2 in WT and Lcn2 Tg (Tg) mice fed a regular chow diet. n=5-7 mice/group. *$p<0.05$ vs WT.

Figure 6

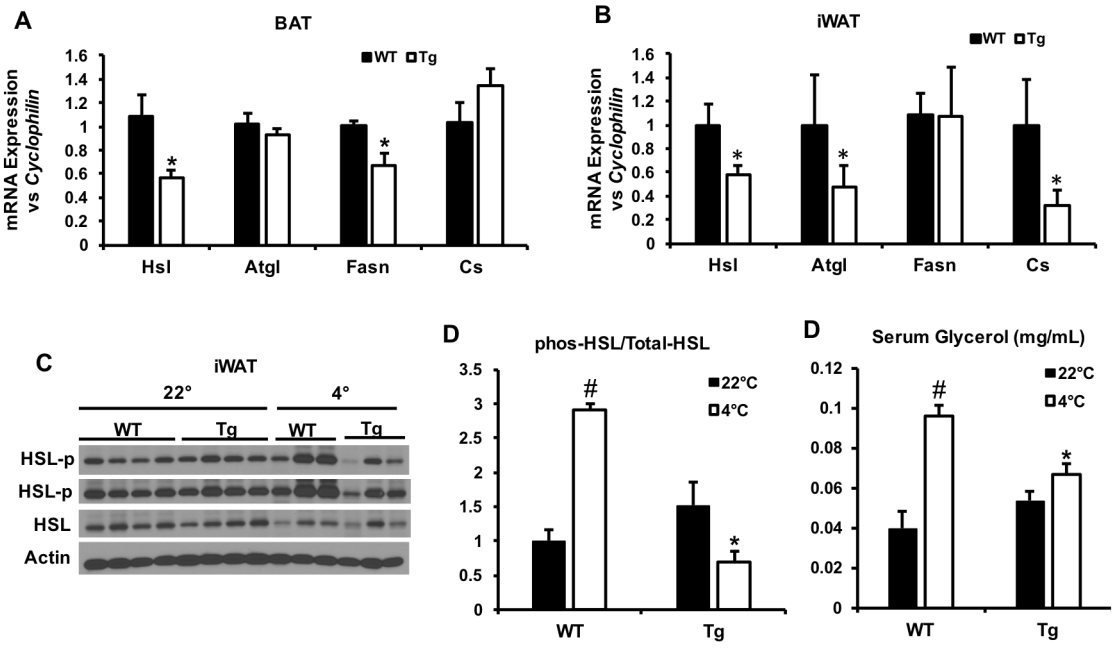
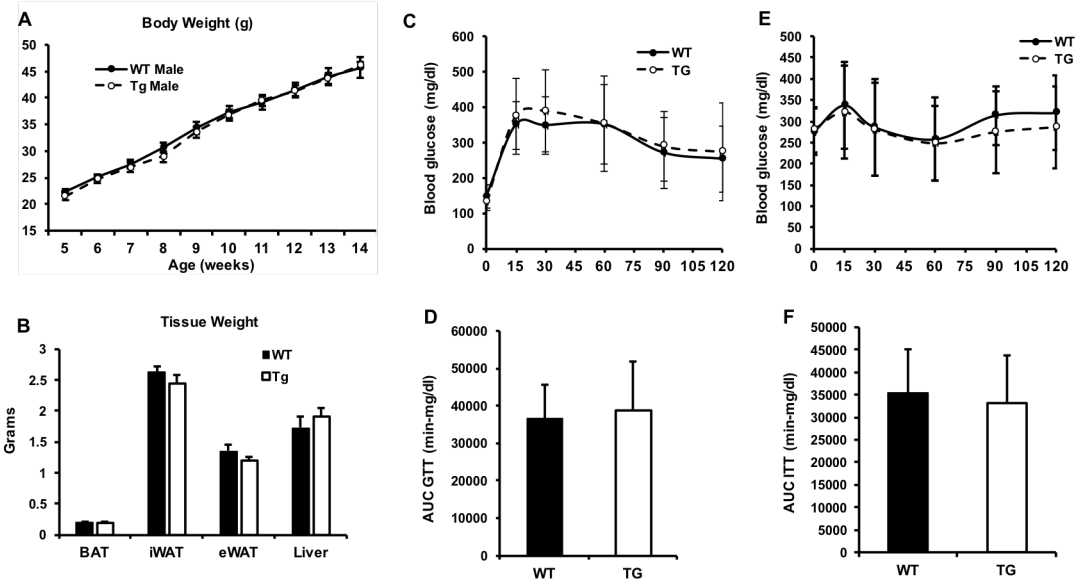


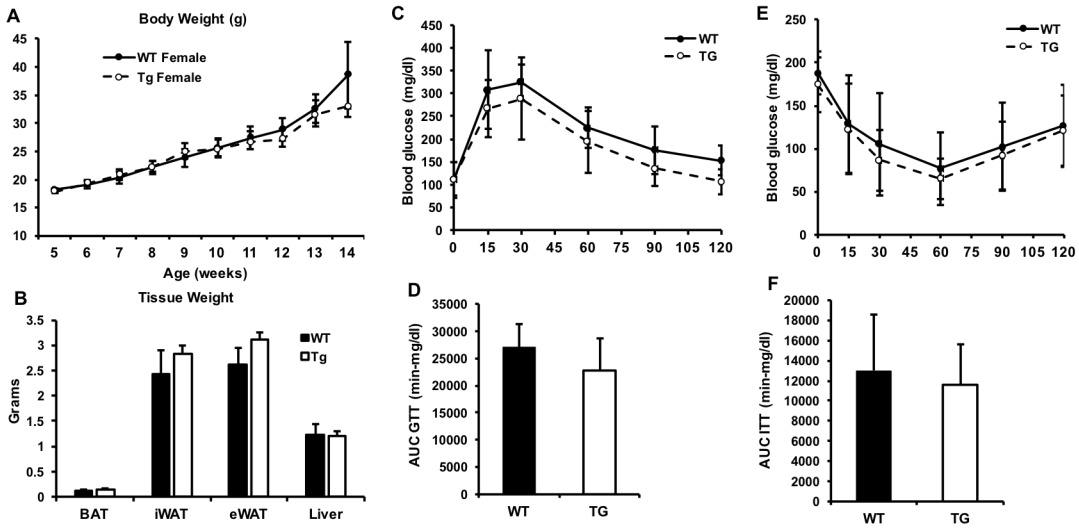
Figure 6: Lipid metabolism in Lcn2 transgenic mice. (A and B) Lipolytic and lipogenic genes in (A) BAT and (B) iWAT from WT and Lcn2 Tg (Tg) mice, n=3-4 mice/group. (C and D) Hormone sensitive lipase (HSL) phosphorylation levels in WT and Lcn2 Tg mice at room temperature (22°C) and following four hours of cold exposure (4°C). (E) Serum glycerol levels in WT and Lcn2 Tg mice at room temperature and following four hours of cold exposure, n=3-4 mice/group. *$p<0.05$ vs WT; #$p<0.05$ vs 22°C.

Supplementary Figure 1



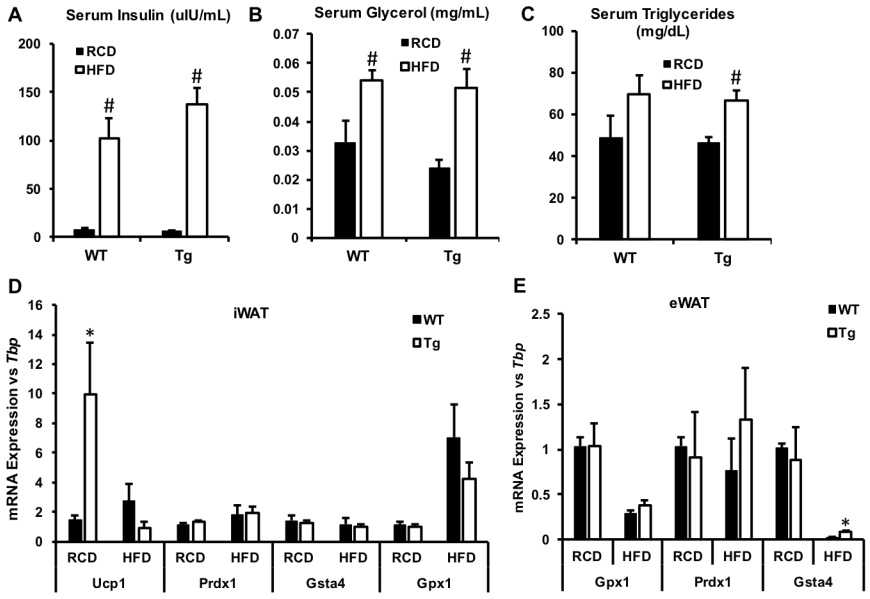
Supplementary Figure 1: Lcn2 transgenic male mice are not protected against diet-induced obesity. (A) Body weight of WT and Lcn2 Tg (Tg) male mice during high-fat diet (HFD) feeding, n=8 mice/group. (B) Tissue weight of WT and Tg male mice following 16 weeks of HFD, n=8 mice/group. (C and D) Glucose tolerance test (GTT) and area under the curve (AUC) in WT and Tg male mice on HFD at 15-16 weeks of age, n=8 mice/group. (E and F) Insulin tolerance test (ITT) and area under the curve (AUC) in WT and Tg male mice on HFD at 16-17 weeks of age, n=8 mice/group.

Supplementary Figure 2



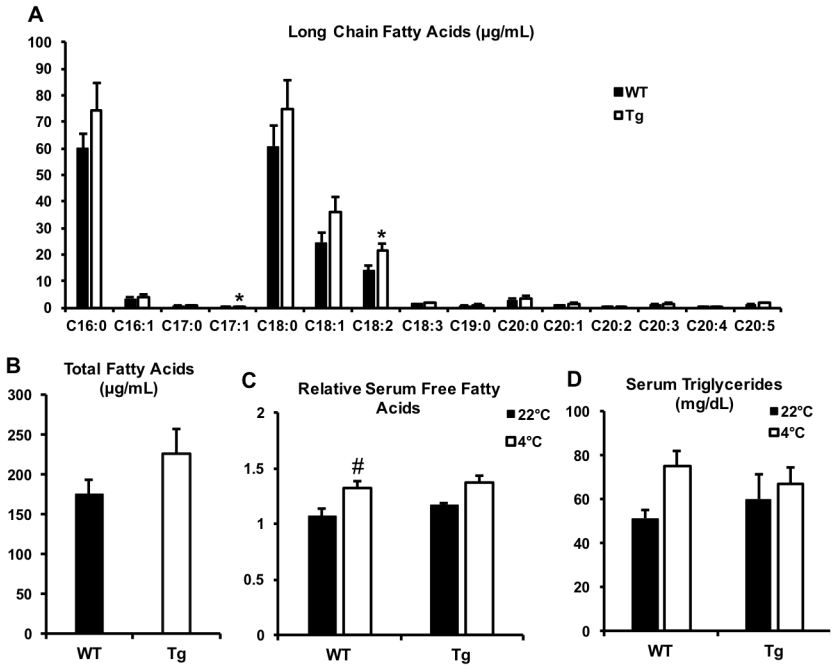
Supplementary Figure 2: Lcn2 transgenic female mice are not protected against diet-induced obesity. (A) Body weight of WT and Lcn2 Tg female mice during high-fat diet (HFD) feeding, n=5-7 mice/group. (B) Tissue weight of WT and Tg female mice following 16 weeks of HFD, n=5-7 mice/group. (C and D) Glucose tolerance test (GTT) and area under the curve (AUC) in female WT and Tg mice on HFD at 15-16 weeks of age, n=5-7 mice/group. (E and F) Insulin tolerance test (ITT) and area under the curve (AUC) in female WT and Tg mice on HFD at 16-17 weeks of age, n=5-7 mice/group.

Supplementary Figure 3



Supplementary Figure 3: Metabolic parameters in Lcn2 transgenic male mice following high-fat diet. (A-C) Serum levels of (A) insulin, (B) glycerol, and (C) triglycerides in WT and Lcn2 Tg (Tg) male mice following 16 weeks of regular-chow diet (RCD) or high-fat diet (HFD), n=3-5 mice/group. (D and E) Expression of oxidative stress genes in (D) iWAT or (E) eWAT from WT and Lcn2 Tg male mice following 16 weeks of regular-chow diet (RCD) or high-fat diet (HFD) feeding, n=3-4 mice/group. #p<0.05 vs RCD; *p<0.05 vs WT of the same treatment group.

Supplementary Figure 4



Supplementary Figure 4: Serum lipid profile in Lcn2 transgenic mice. (A) Long-chain fatty acid profile in serum from WT and Lcn2 Tg (Tg) mice, n=5-7 mice/group. (B) Total serum fatty acids in WT and Tg mice, n=5-7 mice/group. (C) Relative serum free fatty acids and (D) serum triglycerides in WT and Tg mice under room temperature conditions (22°C) and following 4 hours of cold exposure (4°C), n=3-4 mice/group. *p<0.05 vs WT; #p<0.05 vs 22°C.

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Chapter 4

Aging and Improved Healthspan in Lipocalin 2 Transgenic Mice

Abstract

Aging has similar consequences to obesity, in that it increases the risk for development of adipose tissue dysfunction, insulin resistance, dyslipidemia, and liver steatosis. Lipocalin 2 (Lcn2) deficient mice are more prone to diet-induced obesity and metabolic dysfunction, indicating a protective role for Lcn2 in younger mice. In this study, we aged ap2-promoter-driven Lcn2 transgenic (Tg) mice to determine whether overexpressing Lcn2 in adipose tissue can protect against age-related metabolic deterioration. We found decreased adipocyte size in inguinal white adipose tissue (iWAT) from 10-month-old Lcn2 Tg mice relative to WT. This was accompanied by increased markers of adipogenesis in iWAT and attenuation of the age-related decline in AMP-activated protein kinase (AMPK) phosphorylation in adipose tissue depots. In addition to improvements in adipose tissue function, whole-body metabolic homeostasis was maintained in aged Lcn2 Tg mice. This included improved glucose tolerance and reduced serum triglycerides in older Lcn2 Tg mice relative to WT mice. Further, liver morphology and liver cholesterol levels were improved in older Lcn2 Tg mice, alongside a decrease in markers of liver inflammation and fibrosis. Together, this suggests that overexpression of Lcn2 in adipose tissue not only preserves adipose tissue function during aging, but also promotes maintenance of glucose tolerance, decreases dyslipidemia, and prevents liver lipid accumulation and steatosis.

Introduction

Lifespan has increased considerably in developed nations in the past 100 years (1,2). This increase in lifespan has prompted an increase in diseases associated with aging including diabetes, cancer, cardiovascular disease, and neurodegenerative diseases (3-5). Thus, there is now interest in understanding and improving healthspan alongside lifespan.

Aging is associated with perturbations in metabolism including insulin resistance, dyslipidemia, and inflammation (3,5-7). Increasing adiposity and ectopic fat accumulation along with decreased lean mass contribute to the development of insulin resistance in older humans (6,8-10). Changes in the adipose tissue secretory profile, including an increase in secretion of pro-inflammatory cytokines, further contributes to inflammation and insulin resistance (11,12). Unlike white adipose tissue (WAT), the presence and activity of brown adipose tissue (BAT) and beige adipocytes in WAT is decreased in aging (13-17). As brown and beige adipocytes contribute to insulin sensitivity and triglyceride clearance (18,19), this decline may contribute to age-related metabolic deterioration.

On a cellular level, several nutrient sensing pathways have been implicated in the pathology of aging. Signaling through the insulin-like growth factor-1 (IGF-1)/insulin pathway negatively regulates several proteins involved in aging, including sirtuins (20). Further, the mammalian target of rapamycin (mTOR) pathway is activated by insulin and is involved in cell growth, protein synthesis, and lipogenesis. Approaches that inhibit mTOR activity have been shown to increase lifespan and improve insulin sensitivity in mice (21). AMP-activated protein kinase (AMPK) is a cellular energy sensor that is involved in glucose uptake and mitochondrial biogenesis. Treatment with the AMPK-activator metformin have improved longevity and health in mice, indicating increasing

AMPK activity increases healthspan (22). Together, this suggests that nutrient sensing and subsequent regulation of metabolic pathways is integral for maintaining health during aging.

Lipocalin 2 (Lcn2) protein levels are increased in visceral adipose tissue of aged mice and in plasma of older humans, suggesting a role for Lcn2 in aging (23,24). In rodent models, Lcn2 is involved in the development of age-related neurodegenerative diseases including Alzheimer's disease (25,26). Younger mice deficient in Lcn2 have increased inflammation, worsened insulin resistance, and develop liver steatosis, indicating the presence of Lcn2 is protective (27,28). However, whether Lcn2 protects against age-related metabolic diseases in older mice has not been well-studied.

The objective of this study was to determine whether adipose-tissue derived Lcn2 can prevent the deleterious changes in whole-body metabolism that are associated with aging. We utilized both male and female ap2-driven Lcn2 transgenic mice that overexpress Lcn2 in adipose tissue and analyzed markers of metabolic healthspan.

Materials and Methods

Mice Generation and Handling

Ap2-promoter-driven Lcn2 transgenic mice were generated as previously described in chapter 3 of this thesis. Animals were housed at 22°C in a specific pathogen-free facility at the University of Minnesota. Animal studies were conducted with the approval of the University of Minnesota Animal Care and Use Committee and conformed to the National Institute of Health guidelines for laboratory animal care.

For the aging study, WT and Lcn2 transgenic mice from the same litter were housed at 22°C in a 12:12-h light-dark cycle with free access to water. Female and male mice were fed a regular chow diet for the duration of the study. Following an overnight fast, female mice were sacrificed at 18 months of age and male mice were sacrificed at 10 months of age. Tissues and serum were collected and immediately frozen in liquid nitrogen.

Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

GTT and ITT were conducted in the same set of male mice at 16-17 weeks of age and again at 34-35 weeks of age. GTT and ITT were conducted in female mice at 62-63 weeks of age. For GTT, mice were fasted overnight (18 hours) prior to the start of the test. For ITT, mice were fasted for 6 hours prior to the start of the test. Mice were given intraperitoneal glucose (0.75 g/kg) or insulin (0.75 units/kg) injections at time 0. Blood via the tail vein was tested for glucose level using an Ascensia Contour glucometer (Ascensia Diabetes Care, Parsippany, NJ) at 0-, 15-, 30-, 60-, 90-, and 120-minutes following glucose or insulin injection.

Histology

Inguinal white adipose tissues, brown adipose tissues, and liver were fixed in 10% neutral buffered formalin (Thermo Scientific, Rockford, IL) for 24 hours and embedded in paraffin. After deparaffinization and rehydration, tissues were sectioned into 4 μm and stained with hematoxylin and eosin (H&E) using standard techniques. Images were taken under light microscopy at the specified magnification.

Serum Assays

Fasting blood glucose was measured at sacrifice using an Ascensia Contour glucometer (Ascensia Diabetes Care, Parsippany, NJ). Serum insulin was measured using the Mouse Insulin ELISA kit (Thermo Scientific, Frederick, MD). Serum triglycerides were measured using the Stanbio LiquiColor® Triglycerides kit (Stanbio, Boerne, Texas) according to the manufacturer's instructions. Serum glycerol was measured using the Free Glycerol Kit (Sigma, Saint Louis, MO) according to manufacturer's instructions. Serum β-hydroxybutyrate was measured using the Stanbio LiquiColor® Beta-hydroxybutyrate kit (Stanbio, Boerne, Texas) according to the manufacturer's instructions. Serum cholesterol was measured using the Stanbio LiquiColor® Cholesterol enzymatic kit (Stanbio, Boerne, Texas) and total, HDL and LDL cholesterol levels were calculated according to manufacturer's instructions.

Measurement of Triglycerides and Cholesterol in Liver

Lipids were extracted from frozen liver (100 mg) as previously described (27), using the Bligh-Dyer method (29). Triglyceride content was measured using the Stanbio LiquiColor® Triglycerides kit (Stanbio, Boerne, Texas). Cholesterol content was measured using the Stanbio LiquiColor® Cholesterol enzymatic kit (Stanbio, Boerne, Texas).

Statistical analysis

Values are reported as mean +/- standard error of the mean (SEM). Statistical significance was determined by two-tailed Student's t test, where a P value less than 0.05 was considered significant.

Results

1. Lcn2 expression in response to inflammation is decreased in middle-aged mice.

Lcn2 has previously been reported to increase in response to aging in mice and humans (23,24). We measured Lcn2 protein adipose tissue depots and liver from young, 3-month-old mice and older, 10-month-old mice to determine changes in Lcn2 with aging. Lcn2 protein levels were increased in BAT of older mice, but decreased in inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) (Fig. 1A). As Lcn2 is highly inducible in response to inflammation (30), we also measured Lcn2 induction in response to inflammatory stimulation by lipopolysaccharide (LPS) in younger and older mice. Surprisingly, Lcn2 levels in all three adipose tissue depots from older mice had an attenuated response to LPS when compared to the younger mice (Fig. 1A). This raises the question of whether attenuation of Lcn2 in response to aging and inflammatory stimuli contributes to the progression of age-related diseases and whether overexpression of Lcn2 can prevent this.

We utilized ap2-promoter-driven Lcn2 transgenic (Tg) mice to overexpress Lcn2 in adipose tissue. Lcn2 protein levels and gene expression were higher in BAT and iWAT from both 4-month and 10-month old Tg mice when compared to WT mice of the same age (Fig. 1B, 1C, 1D). While Lcn2 gene expression was significantly increased in eWAT from Tg mice when compared to WT mice (Fig. 1E), there was no change in protein levels of Lcn2 in eWAT or serum (Fig. 1B).

2. Body weight and adiposity in middle-aged Lcn2 Tg mice.

To investigate the effect of overexpression of Lcn2 in adipose tissue during aging on metabolic healthspan, we aged male and female WT and Lcn2 Tg mice. Male mice were aged until 10-months, generally equivalent to middle-age in humans. Female mice were aged to 18-months, equivalent to old-age in humans.

Aging is associated with an increase in adiposity, specifically an increase in visceral adiposity (8,9), so we first looked at changes in body composition. Both male and female WT and Lcn2 Tg mice gained similar amounts of body weight with age (Fig. 2A and Supplementary Fig. 1A). In male Lcn2 Tg mice, there was no difference in iWAT, eWAT, or liver weight following aging (Fig. 2B). There was a slight, but significant increase in BAT weight in Lcn2 Tg male mice when compared to WT (Fig. 2B). Aging is associated with a decreased amount of BAT and it is hypothesized that this decrease contributes to age-related metabolic disease (14,15,17), indicating increased BAT mass in older Tg mice may be beneficial. Histology of BAT demonstrated that brown adipocyte size was similar to WT in Lcn2 Tg mice, indicating the increase in BAT mass is not due to adipocyte hypertrophy or whitening of brown fat (Fig. 2C). While thermogenic genes were unchanged in BAT from older Lcn2 Tg mice, there was a trend towards increased expression of estrogen-related receptor alpha (*Errα*), an important regulator of oxidative metabolism (Fig. 2D).

While there was no change in iWAT weight, adipocyte size was markedly smaller following aging in male Lcn2 Tg mice when compared to WT mice (Fig. 2E). This suggests a decrease in age-related white adipocyte hypertrophy in Lcn2 Tg mice. Additionally, there

was a trend towards increased expression of the mitochondrial oxidative metabolism genes cytochrome c oxidase subunit 8b (*Cox8b*), cytochrome c oxidase subunit IV (*CoxIV*), and ATP synthase subunit beta (*Atp5b*) in iWAT from 10-month-old Lcn2 Tg mice (Fig. 2F). Decreased adipocyte hypertrophy and increased mitochondrial activity suggest Lcn2 Tg iWAT is metabolically heathier.

In female mice, there was no difference in overall tissue weight between genotypes. However, female Lcn2 Tg mice had significantly lower visceral (gonadal) adipose tissue weight per gram of body weight (Supplementary Fig. 1B). As accumulation of fat in visceral adipose tissue correlates with age-related insulin resistance and inflammation (10), this could suggest improved metabolic health in aged, female Lcn2 Tg mice.

3. Middle-aged Lcn2 Tg mice have improved adipose tissue metabolic function.

Metabolically healthy adipose tissue has a protective function against age-related complications (7). We next determined if overexpression of Lcn2 in adipose tissue can improve adipose tissue function during aging. Several metabolic pathways have been implicated in aging (6). Activating AMPK has been reported to promote healthspan and longevity (6,22). We measured AMPK phosphorylation in adipose tissue from fasted WT and Lcn2 Tg male mice. In younger mice, there was no difference in phosphorylation of AMPK in BAT, eWAT or iWAT when comparing genotypes (Fig. 3A-3F). Following aging, AMPK phosphorylation levels decline in WT mice. However, decreased AMPK phosphorylation was attenuated in BAT, eWAT, and iWAT from Lcn2 Tg mice.

Decline in adipogenic capacity of subcutaneous adipose tissue is a major contributor to age-related pathologies, including insulin resistance and lipid spillover into circulation and non-adipose tissues (7). In iWAT from older Lcn2 Tg mice, we found increased levels of peroxisome proliferator-activated receptor gamma (PPARγ) and adiponectin relative to WT mice (Fig. 3E, 3F). This could be suggestive of improved adipogenesis in adipose tissue in older Lcn2 Tg mice. Consistent with improved adipogenesis, decline in *Glut4* expression following aging was significantly attenuated in iWAT and eWAT from Lcn2 Tg mice. Together this data indicates improved adipose tissue function in older Lcn2 Tg mice, which may have implications for whole-body metabolic homeostasis.

4. Middle-aged Lcn2 Tg mice have improved glucose tolerance.

Alterations in adipose tissue function and distribution during aging contribute to insulin resistance and glucose intolerance (31). Therefore, we examined whether overexpression of Lcn2 during aging modifies glucose homeostasis. Glucose and insulin tolerance tests (GTT and ITT) were conducted at 3 months and 10 months of age in WT and Lcn2 Tg male mice fed a RCD. In younger, male Lcn2 Tg mice, there was no difference in glucose tolerance or insulin sensitivity when compared to WT mice (Fig. 4A-4D). However, in older Lcn2 Tg mice, area under the curve following glucose administration was significantly lower than WT mice, indicating improved glucose tolerance (Fig. 4E, 4F). This suggests that overexpression of Lcn2 in adipose tissue mitigates the development of glucose tolerance during aging. Despite improved GTT, there was no difference in insulin sensitivity between WT and Lcn2 Tg mice at 10 months of age (Fig. 4G, 4H).

Consistent with improved glucose tolerance, Lcn2 Tg mice had decreased fasting blood glucose levels at 10 months of age (Fig. 4I). This was accompanied by a trend towards increased serum insulin levels (Fig. 4J). The decrease in fasting glucose and improved glucose tolerance could potentially be due to the increase in serum insulin in Lcn2 Tg mice, leading to increased insulin-stimulated glucose uptake despite unchanged insulin sensitivity.

5. *Serum lipid profile in middle-aged Lcn2 Tg mice.*

In response to the age-related decline in adipogenesis and decreased ability to store lipids in adipose tissue, circulating levels of lipids increase (5,7). This increase in circulating lipids is a contributing factor to ectopic fat accumulation and the development of insulin resistance (7). We measured serum levels of lipids in WT and Lcn2 Tg male mice following aging. Serum triglycerides were significantly lower in 10-month-old Lcn2 Tg mice relative to WT, which could indicate Lcn2 protects against age-related dyslipidemia (Fig. 5A). We also measured serum glycerol as a marker of free fatty acids and serum β-hydroxybutyrate and found no difference between WT and Lcn2 Tg mice (Fig. 5B-5C). Moreover, there was no difference in serum cholesterol, which is known to resist change in mice (Fig. 5C-5F).

6. *Middle-aged Lcn2 Tg mice have decreased liver lipid accumulation and improved liver metabolic health.*

Aging is associated with increased circulating lipids, which results in ectopic fat accumulation in non-adipose tissues (7). We saw increased adipogenic markers, improved adipose tissue function, and significantly lower levels of serum triglycerides in Lcn2 Tg

mice. This led us to hypothesize that Lcn2 Tg mice may have less age-related lipid accumulation and improved liver health. Therefore, we next evaluated liver lipid accumulation and metabolism in aged mice. Morphologically, liver sections from 10-month-old Lcn2 Tg mice appeared to have less lipid droplet accumulation when compared to WT mice (Fig. 6A). Analysis of tissue lipid levels showed no change in liver triglyceride levels (Fig. 6B). On the other hand, liver cholesterol levels were significantly decreased in Lcn2 Tg mice (Fig. 6C). Consistent with decreased liver cholesterol levels, genes involved in cholesterol synthesis were also decreased in Lcn2 Tg liver relative to WT (Fig. 6D). Lipogenic genes were significantly down-regulated in Lcn2 Tg liver (Fig. 6E), which could contribute to the decreased lipid droplet accumulation. Expression of phosphoenolpyruvate carboxykinase 1 (*Pck1*), a major regulator of gluconeogenesis, was also decreased in Tg mice (Fig. 6F).

Decreases in lipid accumulation in liver suggest improved liver function. Indeed, we saw decreases in the expression of genes involved in oxidative stress, inflammation, and liver fibrosis in Lcn2 Tg liver (Fig. 6G and 6H). Together, this suggests that overexpression of Lcn2 in adipose tissue can promote liver health, which may further contribute to the improvements in whole-body glucose and lipid homeostasis seen in middle-aged Lcn2 Tg mice.

Discussion

Previous studies have found Lcn2 prevents diet-induced obesity, insulin resistance, liver steatosis and inflammation in young mice (27,28). Similar to obesity, aging also increases the risk for insulin resistance, dyslipidemia, impaired nutrient

signaling, and liver dysfunction (5,6,10). We therefore hypothesized that Lcn2 may also be able to prevent age-related metabolic deterioration. Utilizing ap2-promoter-driven Lcn2 Tg mice, we found improvements in key metabolic factors that decline with age including adipose tissue function, whole-body glucose and lipid homeostasis, and liver function.

Increasing AMPK activity through genetic or pharmacological means has a beneficial effect on glucose and lipid metabolism, leading to improved healthspan and longevity (6,22). In WT mice, we saw a decline in AMPK phosphorylation in adipose tissue depots of older mice when compared to younger mice. This decrease in AMPK phosphorylation was attenuated in older Lcn2 Tg mice. AMPK activity in adipose tissue has been reported to inhibit lipogenesis and promote fat oxidation (32). Further, AMPK regulates peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) to induce mitochondrial biogenesis in adipose tissue (33). While we saw no change in *Pgc-1α* or oxidative gene expression in older Lcn2 Tg mice, it is more likely that AMPK is regulating energy metabolism at the protein level through post-translational modifications. Further, whether increased AMPK phosphorylation in Lcn2 Tg adipose tissue is a cause or effect of changes in whole-body metabolism remains unclear.

There is an age-related decline in PPARγ in adipose tissue, leading to reduced adipogenic capacity (7,34). Impairment of adipogenesis in subcutaneous adipose tissue during aging is a major contributor to lipid spillover into circulation and non-adipose tissues (7). In older Lcn2 Tg mice, there was an increase in adipogenic markers, including increased PPARγ and adiponectin levels in iWAT. This was further reflected in

an increase in the PPARγ target gene, *Glut4*, in iWAT from Lcn2 Tg mice. In rodent studies, treatment with the PPARγ-agonist troglitazone resulted in smaller adipocyte size and reduced plasma triglyceride level, with no change in overall adipose tissue mass (35). Consistent with this, we saw smaller adipocyte size in iWAT and decreased serum triglyceride levels in older Lcn2 Tg mice, despite no difference in WAT weight. This suggests that increased PPARγ and improved adipose tissue function in Lcn2 Tg mice may have implications for whole-body energy homeostasis.

Lcn2 Tg mice had significantly improved GTT when compared to WT mice, indicating overexpression of Lcn2 in adipose tissue attenuates the age-related decline in glucose tolerance. At the same time, there was no difference in insulin sensitivity in Lcn2 Tg mice relative to WT. Enhanced glucose tolerance could therefore be a result of the increase in serum insulin levels in Tg mice. We also saw changes in insulin-responsive genes in Tg mice, including modulation of the gluconeogenic gene *Pck1* in liver and increased *Glut4* expression in WAT. In agreement with our data, a recent study by *Mosialou et al* demonstrated increased insulin levels in mice treated chronically with Lcn2 and enhanced insulin secretion from pancreatic islets treated with Lcn2 (36). Together, this suggests that Lcn2 from adipose tissue may potentiate insulin secretion, which suppresses hepatic gluconeogenesis and increases glucose transport to prevent a decline in glucose tolerance during aging.

Interestingly, while glucose tolerance was improved in middle-aged, male Lcn2 Tg mice versus WT, we saw no difference in older, female Lcn2 Tg mice. We have previously reported gender differences in regards to Lcn2 levels in aging mice, with

female, old mice expressing much higher levels of Lcn2 in subcutaneous WAT and lower levels of Lcn2 in visceral WAT relative to male, old mice (23). Therefore, the sexual dimorphism in glucose homeostasis could be due to gender differences in Lcn2 distribution with aging.

Advancing age is a risk factor for the development of non-alcoholic fatty liver disease (NAFLD), marked by liver lipid accumulation leading to fibrosis and inflammation (37). During aging, decreased capacity for lipid storage in subcutaneous adipose tissue results in lipid accumulation in liver (7). Further, circulating factors secreted from adipose tissue play a role in the development of NAFLD (38). In middle-aged Lcn2 Tg mice, we found less apparent lipid droplets in liver relative to WT mice. Further, cholesterol levels and genes involved in cholesterol synthesis were decreased in liver from Lcn2 Tg mice. Decreased liver cholesterol in Lcn2 Tg mice may indicate a decrease in the manufacture and secretion of very-low density lipoprotein (VLDL) for exportation of triglycerides to peripheral tissues, which could further explain the decrease in serum triglycerides. Consistent with a decrease in lipid accumulation in the liver, we saw a decrease in gene markers of oxidative stress, inflammation, and fibrosis. This suggests that overexpression of Lcn2 in adipose tissue protects from liver lipid accumulation and further liver injury and inflammation.

In conclusion, attenuation of the age-related decline in AMPK phosphorylation and adipogenic markers in adipose tissue of Lcn2 Tg mice suggests improved adipose tissue health in response to overexpression of Lcn2. We saw improvements in glucose tolerance, serum lipids and liver health in Lcn2 Tg mice, indicating that adipose-derived Lcn2 not only effects adipose tissue, but also can regulate metabolism in other tissues.

We did not see changes in serum levels of Lcn2 in Tg mice, raising the question of whether Lcn2 directly regulates other tissues or indirectly regulates them by improving adipose tissue function. Together, these results indicate that Lcn2 overexpression in adipose tissue promotes metabolic healthspan.

Figure 1

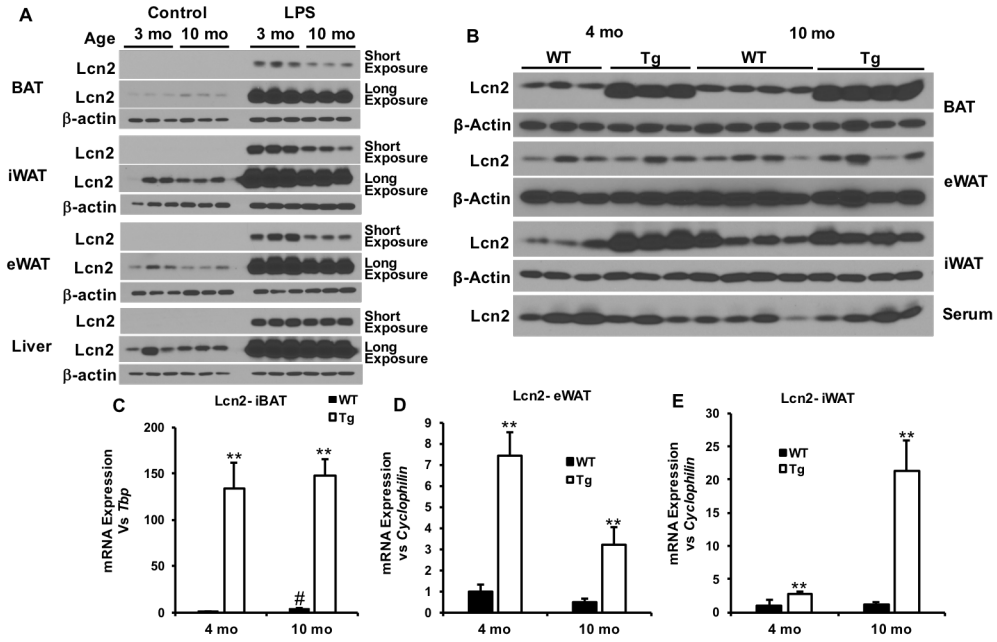


Figure 1: Lcn2 expression in response to aging and inflammation. (A) Lcn2 levels in brown adipose tissue (BAT), inguinal white adipose tissue (iWAT), epididymal adipose tissue (eWAT) and liver at 3 months (3 mo) or 10 months (10 mo) of age under basal or lipopolysaccharide (LPS)-treated conditions in wild-type (WT) male mice. (B) Lcn2 levels in adipose tissue from WT or Lcn2 transgenic (Tg) male mice at 4 months (4 mo) or 10 months (10 mo) of age. (C-E) Lcn2 gene expression in (C) BAT, (D) eWAT, and (E) iWAT from WT and Lcn2 Tg male mice at 4 mo or 10 mo of age, n=3-5 mice/group. #$p<0.05$ vs WT 4 mo; **$p<0.01$ vs WT of same age.

Figure 2

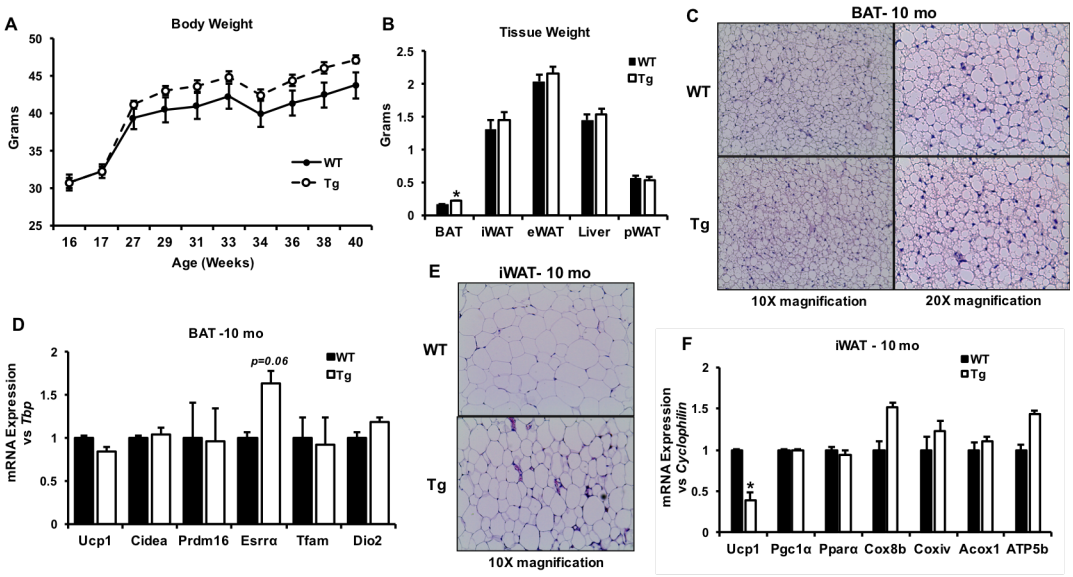


Figure 2: Body weight and adiposity in middle-aged Lcn2 transgenic mice. (A) Body weight in wild-type (WT) and Lcn2 transgenic (Tg) male mice during aging, n=6-8 mice/group. (B) Tissue weight in WT and Lcn2 Tg male mice at sacrifice at 10 months of age, n=6-8 mice/group. (C) H&E staining of brown adipose tissue (BAT) sections from 10-month-old (10 mo) WT and Lcn2 Tg male mice. (D) Gene expression in BAT from 10-month-old WT and Lcn2 Tg male mice, n=3-4/group. (E) H&E staining of inguinal white adipose tissue (iWAT) sections from 10-month-old WT and Lcn2 Tg male mice. (F) Gene expression in iWAT from 10-month-old WT and Lcn2 Tg male mice, n=3-5/group. *p<0.05 vs WT.

Figure 3

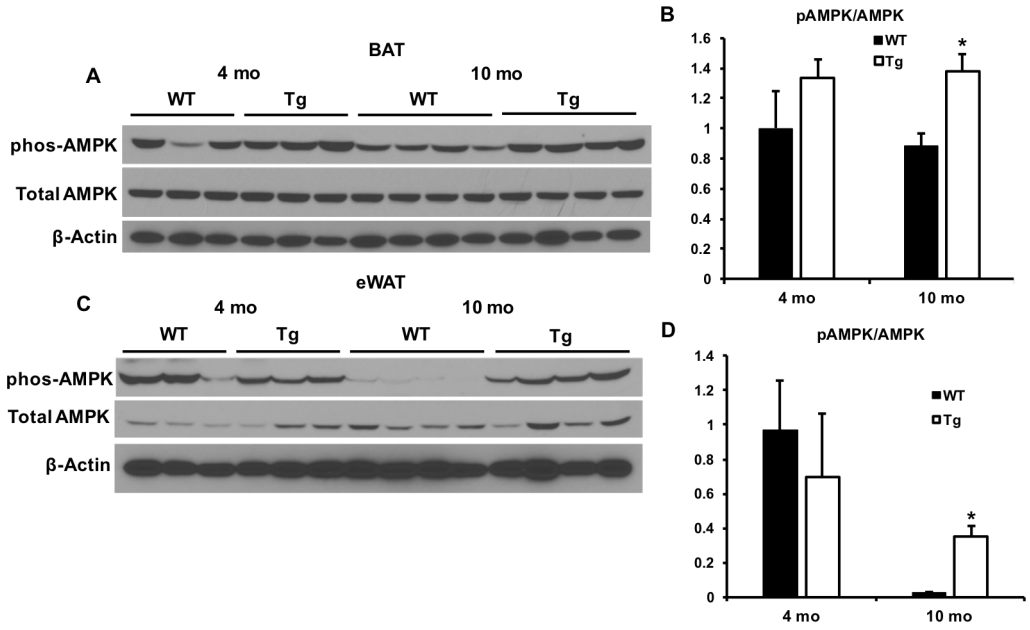


Figure 3: AMPK phosphorylation and adipogenic markers in adipose tissue from middle-aged mice. (A-D) AMPK phosphorylation levels and quantification (fold change) in (A, B) brown adipose tissue (BAT) and (C, D) epididymal white adipose tissue (eWAT) from 4-month (4 mo) and 10-month (10 mo) WT and Lcn2 transgenic (Tg) male mice. *p<0.05 vs WT of same age.

Figure 3 continued

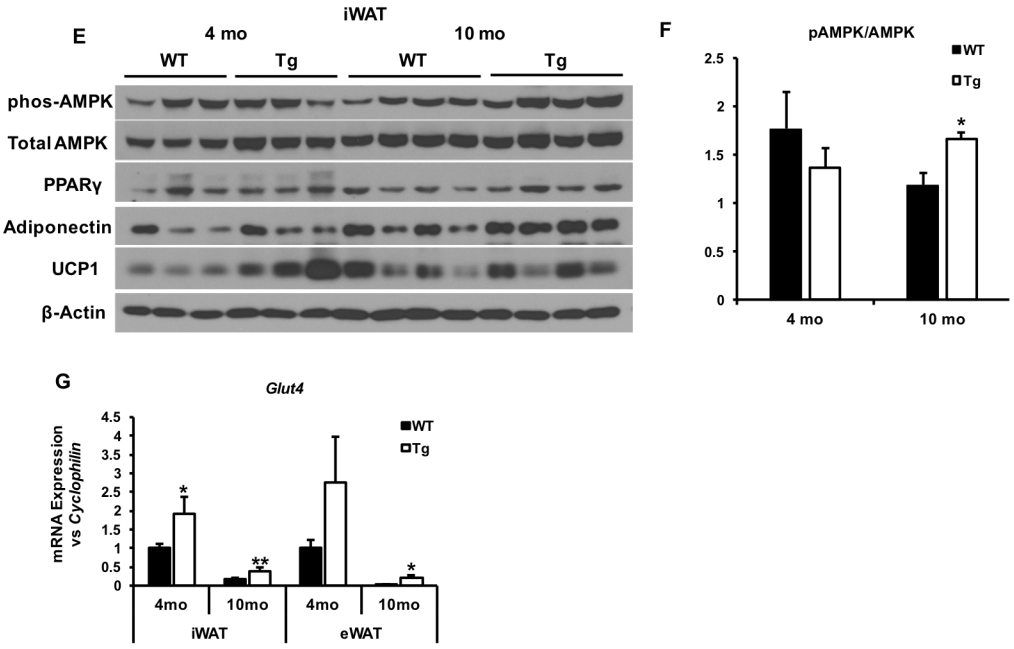


Figure 3 continued: AMPK phosphorylation and adipogenic markers in adipose tissue from middle-aged mice. (E) AMPK phosphorylation levels and adipogenic markers in inguinal white adipose tissue from 4-month and 10-month-old WT and Lcn2 Tg male mice. (F) Quantification of AMPK phosphorylation in iWAT. (G) Glut 4 gene expression in iWAT and eWAT from 4-month and 10-month-old WT and Lcn2 Tg male mice, n=3-5 mice/group. *$p<0.05$ vs WT of same age; **$p<0.01$ vs WT of same age.

Figure 4

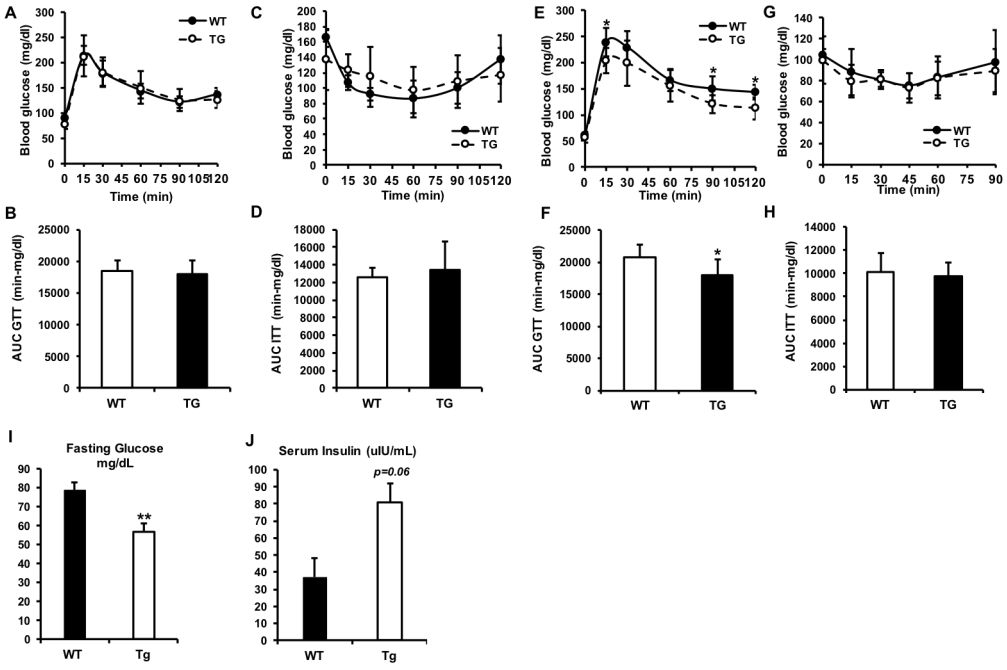


Figure 4: Glucose homeostasis in Lcn2 transgenic mice. (A, B) Glucose tolerance test (GTT) and area under the curve (AUC) in 4-month-old WT and Lcn2 transgenic (Tg) male mice, n=6-8 mice/group. (C, D) Insulin tolerance test (ITT) and AUC in 4-month-old WT and Lcn2 Tg male mice, n=6-8 mice/group. (E, F) GTT and AUC in 10-month-old WT and Lcn2 Tg male mice, n=6-8 mice/group. (G, H) ITT and AUC in 10-month-old WT and Lcn2 Tg male mice, n=6-8 mice/group. (I) Blood glucose in WT and Lcn2 Tg male mice following overnight fasting, n=6-8 mice/group. (J) Insulin levels in serum collected from WT and Lcn2 Tg male mice following overnight fasting, n=6-8 mice/group. *p<0.05 vs WT; **p<0.01 vs WT.

Figure 5

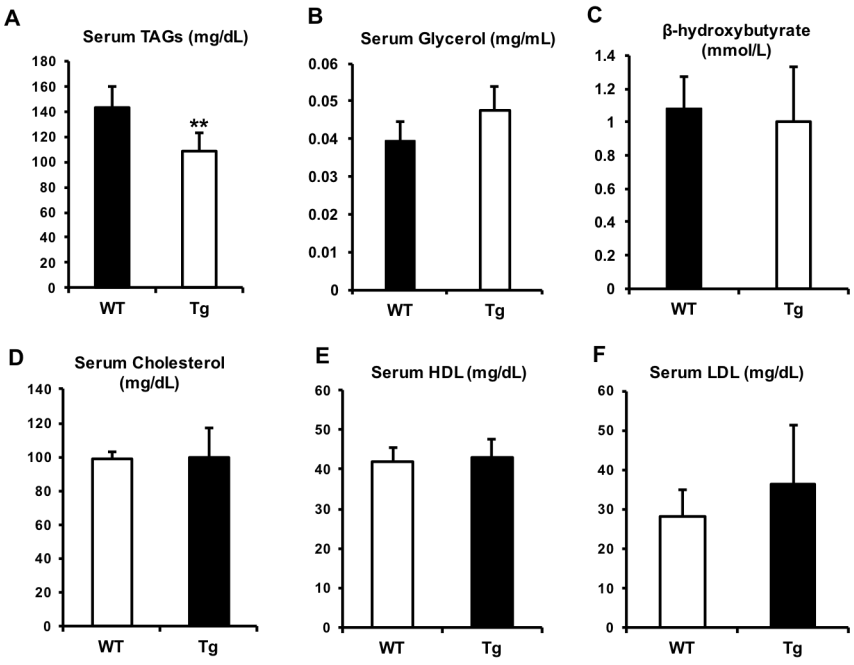


Figure 5: Serum lipid profile in middle-aged Lcn2 transgenic mice. (A) Triglycerides (TAGs), (B) glycerol, (C) β-hydroxybutyrate, (D) total cholesterol, (E) HDL cholesterol, and (F) LDL cholesterol in serum from 10-month-old WT and Lcn2 transgenic (Tg) male mice following overnight fasting, n=6-8 mice/group. **p<0.01 vs WT.

Figure 6

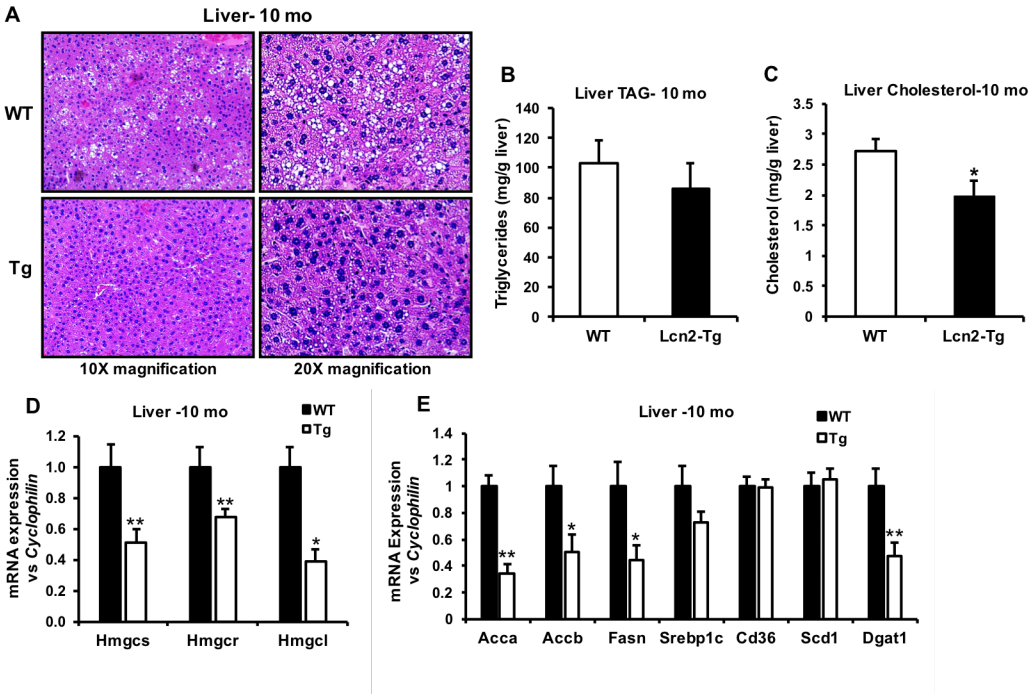


Figure 6: Liver lipid accumulation in middle-aged Lcn2 transgenic mice. (A) H&E staining of liver sections from 10-month-old WT and Lcn2 transgenic (Tg) male mice. (B) Triglyceride (TAG) level and (C) cholesterol levels in liver from 10-month-old WT and Lcn2 Tg male mice, n=6-8 mice/group. (D) Cholesterol synthesis gene expression and (E) lipogenic gene expression in liver from 10-month-old WT and Lcn2 Tg male mice, n= 5-6 mice/group. *p<0.05 vs WT; **p<0.01 vs WT.

Figure 6 continued

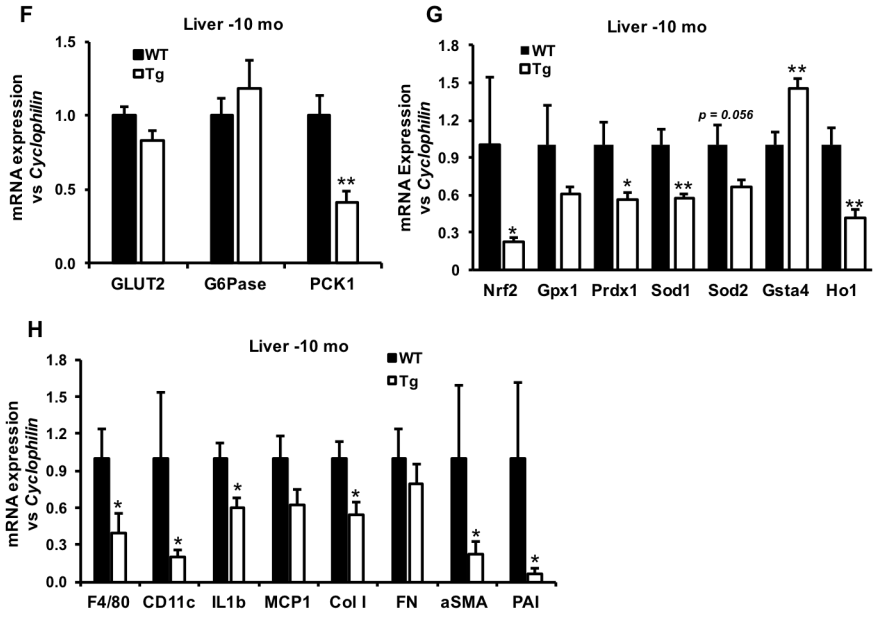


Figure 6 continued: Liver lipid accumulation in middle-aged Lcn2 transgenic mice. (F) Gluconeogenic gene expression, (G) oxidative stress gene expression, and (H) inflammation and fibrosis gene expression in liver from 10-month-old WT and Lcn2 Tg male mice, n=5-6 mice/group. *p<0.05 vs WT; **p<0.01 vs WT.

Figure 7

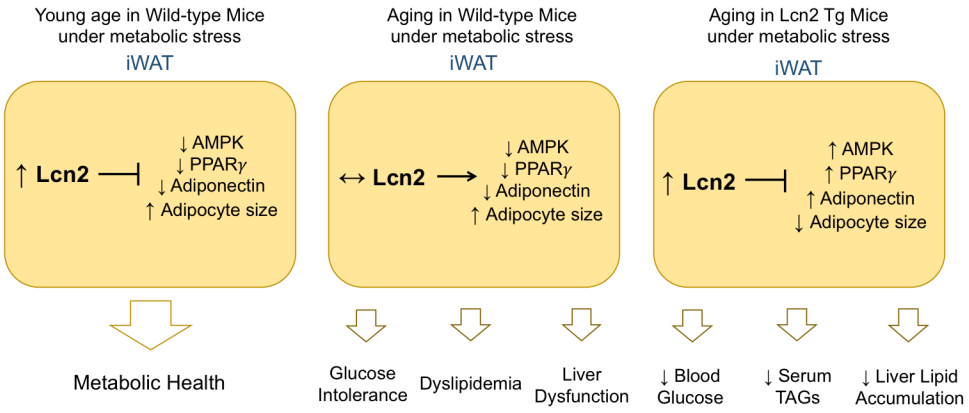
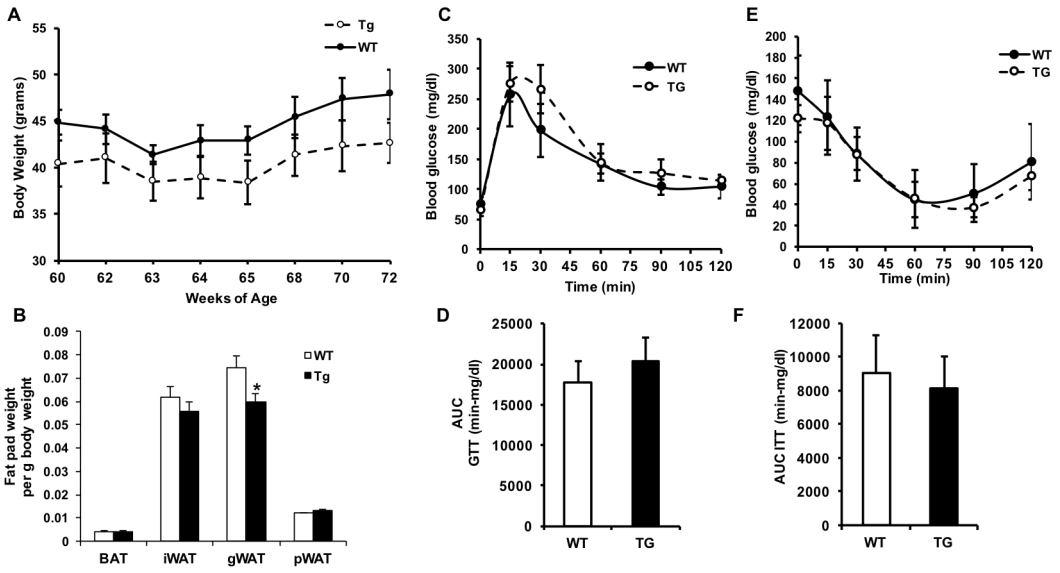


Figure 7: Aging in Lcn2 transgenic mice.

Supplementary Figure 1



Supplementary Figure 1: Aging in female Lcn2 transgenic mice.
(A) Body weight of WT and Lcn2 transgenic (Tg) female mice, n= 4 mice/group. (B) Tissue weight normalized to body weight in 18-month-old WT and Lcn2 Tg female mice, n= 4 mice/group. (C and D) Glucose tolerance test (GTT) and area under the curve (AUC) in WT and Lcn2 Tg female mice at 15 months of age, n= 4 mice/group. (E and F) Insulin tolerance test (ITT) and AUC in WT and Lcn2 Tg female mice at 15 months of age, n=4 mice/group. *$p<0.05$ vs WT.

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Conclusions and Future Directions

Lipocalin 2 (Lcn2) is an adipose tissue-derived secreted protein that has previously been shown to regulate cold adaptation and thermogenesis in brown adipose tissue (BAT) (1,2). In the second chapter of this thesis, we determined whether Lcn2 was necessary for thermogenic programming in inguinal white adipose tissue (iWAT). We found that Lcn2 is an effector of retinoic acid (RA)-induced activation of beiging in inguinal adipocytes, particularly in the presence of insulin. Lcn2 was highly expressed in the plasma membrane fraction from adipocytes, suggesting that Lcn2 binds to the plasma membrane to exert its effects. Following insulin treatment, levels of retinoic acid receptor-alpha (RAR-α) on the plasma membrane were increased, and this increase was attenuated in Lcn2 deficient adipocytes. We speculate that Lcn2 may regulate RA-induced thermogenesis by binding to and promoting translocation of RAR-α to the plasma membrane. More research is needed to confirm this hypothesis, and to determine whether Lcn2 can regulate other cell signaling events in a similar manner.

In the third chapter of this thesis, we investigated whether overexpression of Lcn2 in adipose tissue can promote thermogenesis and adipose tissue health. We generated ap2-promoter-driven Lcn2 transgenic (Tg) mice that overexpress Lcn2 in adipose tissue. Overexpression of Lcn2 improved cold adaptation and increased markers of beiging in iWAT. Lcn2 Tg mice had a trend towards increased fat utilization, with a decrease in adipocyte size in iWAT. An interesting finding was a trend towards increased serum levels of the short-chain fatty acid propionate. Several recent studies have identified intestinally-derived Lcn2 as a regulator of gut microbiota composition (3,4). This raises a question of whether Lcn2 derived from adipose tissue can regulate gut health, either directly or indirectly.

Finally, we explored whether overexpression of Lcn2 in adipose tissue prevents age-related metabolic deterioration. Lcn2 Tg mice had increased markers of adipogenesis in adipose tissue, alongside a marked decreased in inguinal adipocyte size. Beyond improving adipose tissue health, overexpression of Lcn2 also maintained glucose tolerance, increased serum insulin, lowered serum triglycerides and promoted markers of liver health in aging mice. This suggests that Lcn2 regulates cross-talk between adipose tissue and other tissues including pancreas and liver. Serum levels of Lcn2 were not increased in Lcn2 Tg mice, leading us to speculate that adipose tissue Lcn2 may not directly bind to and regulate other tissues. Future research could focus on whether Lcn2 promotes whole-body metabolism simply through improving adipose tissue function, whether it regulates secretion of other adipokines, or whether Lcn2 itself has endocrine effects on other tissues.

In conclusion, adipose-tissue derived Lcn2 has effects on both adipose tissue health and whole-body energy homeostasis. This is particularly true under conditions of metabolic stress, including cold and aging. In this thesis and previous studies (5-8), Lcn2 was found to regulate signaling events in response to molecules such as insulin and retinoic acid, indicating Lcn2 may primarily function as an effector of nutrient and hormone signaling.

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